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Award Number: DAMD17-97-1-7183

TITLE: The Role of VDR in Phosphorylation in Vitamin D-Induced Apoptosis

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REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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**REPORT DOCUMENTATION PAGE**Form Approved  
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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jul 98 - 30 Jun 01)	
<b>4. TITLE AND SUBTITLE</b> The Role of VDR in Phosphorylation in Vitamin D-Induced Apoptosis			<b>5. FUNDING NUMBERS</b> DAMD17-97-1-7183	
<b>6. AUTHOR(S)</b> Carmen Narvaez Doctor JoEllen Welsh				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> The University of Notre Dame Notre Dame, Indiana 46556-5612  E-Mail: narvaez.2@nd.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b>  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Report contains color				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				<b>12b. DISTRIBUTION CODE</b>
<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> Vitamin D <sub>3</sub> compounds offer an alternative approach to anti-hormonal therapies for human breast cancer. 1,25-Dihydroxyvitmain D <sub>3</sub> (1,25-D <sub>3</sub> ) acts through the nuclear vitamin D receptor (VDR), a phosphoprotein, which can be phosphorylated by protein kinase C (PKC) and casein kinase II to modulate gene expression. Our lab has shown that 1,25-D <sub>3</sub> induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome <i>c</i> release, and production of reactive oxygen species (ROS). Fluorescence microscopy showed direct evidence of Bax translocation to the mitochondria after treatment with 1,25-D <sub>3</sub> in MCF-7 cells. TPA (a PKC activator) did not induce cytochrome <i>c</i> release or Bax translocation, thus demonstrating that it has no effect on mitochondria. However, when the MCF-7 <sup>D3Res</sup> cells (a vitamin D <sub>3</sub> -resistant variant) were treated with 1,25-D <sub>3</sub> in the presence of TPA, the cells displayed apoptotic morphology and redistribution of both cytochrome <i>c</i> and Bax. The observation that the phorbol ester TPA can sensitize the vitamin D <sub>3</sub> -resistant variant to the effects of 1,25-D <sub>3</sub> suggests an important role for phosphorylation in dictating sensitivity to vitamin D <sub>3</sub> mediated apoptosis. This study indicates that the effects of 1,25-D <sub>3</sub> on mitochondrial disruption might be sensitized through activators of PKC.				
<b>14. SUBJECT TERMS</b> Breast Cancer, Vitamin D <sub>3</sub> , apoptosis, TPA, phosphorylation			<b>15. NUMBER OF PAGES</b> 40	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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## INTRODUCTION

1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, is not only a powerful regulator of calcium homeostasis, but is a steroid hormone with important roles in cell growth and differentiation. 1,25-D<sub>3</sub> is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo*. A variety of synthetic vitamin D analogs that induce breast tumor regression in animals are now undergoing clinical trials in human patients. Our lab has shown that inhibition of breast cancer cell growth in response to 1,25-D<sub>3</sub> involves activation of apoptosis (Welsh *et al.*, 1995; Narvaez and Welsh, 1997). 1,25-D<sub>3</sub> acts through the nuclear vitamin D receptor (VDR), a phosphoprotein, which can be phosphorylated by protein kinase C (PKC) and casein kinase II to modulate gene expression. The main objective of this USAMRC proposal was to assess whether phosphorylation of the VDR affects specific DNA binding and transactivation ability of the VDR in MCF-7 cells in relation to apoptosis following treatment with 1,25-D<sub>3</sub> or TPA (a PKC activator). In addition to examining phosphorylation of the VDR, we investigated the 1,25-D<sub>3</sub> signaling pathway in order to identify specific intracellular events involved in 1,25-D<sub>3</sub> mediated apoptosis and to characterize events which are blocked in MCF7<sup>D3Res</sup> cells (a vitamin D<sub>3</sub>-resistant variant). In particular, the effects of 1,25-D<sub>3</sub> on mitochondrial function and caspase activation were studied. Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c*, and production of reactive oxygen species (Green and Reed, 1998; Susin *et al.*, 1998). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X<sub>L</sub>, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax that lead to its translocation to the mitochondria (Tsujimoto and Shimizu, 2000). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell. We also examined the effects of 1,25-D<sub>3</sub> on MCF-7<sup>D3Res</sup> cells in comparison to the parental MCF-7 cells to determine events that contribute to resistance. The observation that the phorbol ester TPA can potentiate the effects of 1,25-D<sub>3</sub> on induction of apoptosis in breast cancer cells, and more significantly, that TPA can partially sensitize the vitamin D<sub>3</sub> resistant variant to the effects of 1,25-D<sub>3</sub> suggests an important role for phosphorylation in dictating sensitivity to vitamin D<sub>3</sub> mediated apoptosis (Narvaez and Welsh, 1997). This indicates that the effects of 1,25-D<sub>3</sub> on mitochondrial disruption and caspase activity might be sensitized through activators of PKC. These data will help in evaluating the interactions between phosphorylation pathways and vitamin D mediated apoptosis of breast cancer cells.

## RESEARCH ACCOMPLISHMENTS

In the previous Annual Report, I reported that 1,25-D<sub>3</sub> induces apoptosis in MCF-7 cells by disruption of mitochondrial function (a first observation) which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, I demonstrated that Bax translocation and mitochondrial disruption do not occur after 1,25-D<sub>3</sub> treatment of the vitamin D<sub>3</sub> resistant variant, MCF-7<sup>D3Res</sup> cells. The mitochondrial effects of 1,25-D<sub>3</sub> do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor zVAD.fmk. Although caspase inhibition blocks 1,25-D<sub>3</sub> mediated events downstream of mitochondria (such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation), MCF-7 cells still execute apoptosis in the presence of zVAD.fmk, indicating that the commitment to 1,25-D<sub>3</sub> mediated cell death is caspase-independent. These data have now been published, Narvaez, CJ and

Welsh, J (2001) Role of mitochondria and caspases in vitamin D mediated apoptosis of MCF-7 breast cancer cells, *Journal of Biological Chemistry* **276**:9101-9107.

During the second year of the award, I expanded my initial observations and demonstrated that mitochondrial disruption occurs prior to activation of downstream caspases. Redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 hrs of 1,25-D<sub>3</sub> treatment in MCF-7 cells, prior to the onset of apoptotic morphology. Long-term exclusion of cytochrome *c* from the electron transport chain can lead to ROS generation due to incomplete reduction of molecular oxygen. Time course studies demonstrated that ROS production was enhanced within 72 hrs of 1,25-D<sub>3</sub> treatment in MCF-7 cells (Figure 1, *Left*) whereas loss of mitochondrial membrane potential was a late event during 1,25-D<sub>3</sub> mediated apoptosis, (observed after 96 hrs, Figure 1, *Right*). Cytochrome *c* release

into the cytosol triggers caspase activity, which leads to substrate cleavage, and externalization of phosphatidylserine. The earliest detection of phosphatidylserine exposure by binding to annexin V-FITC was 96 hrs after treatment with 1,25-D<sub>3</sub> indicating that mitochondrial disruption precedes activation of downstream caspases. Our results demonstrate that 1,25-D<sub>3</sub> mediates apoptosis of MCF-7 cells through mitochondrial signaling and ROS generation, which is regulated by the Bcl-2 family of apoptotic regulators (Narvaez and Welsh, 2001; Narvaez *et al.*, 2001). Caspases

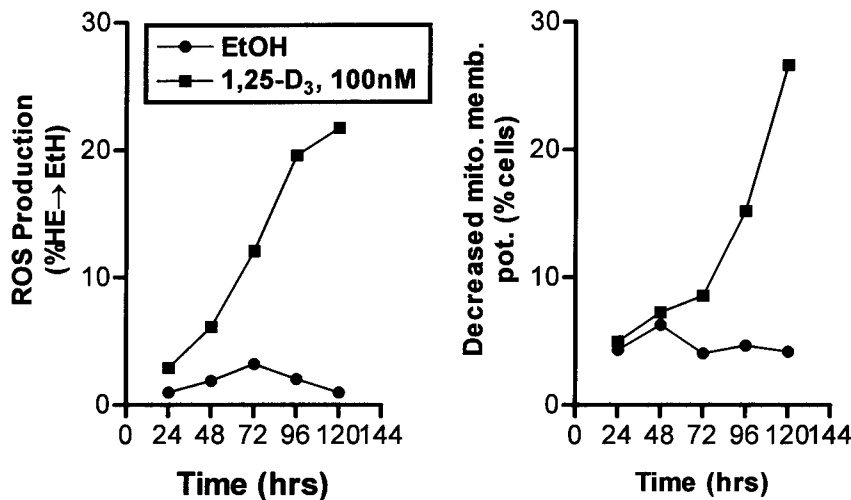


Figure 1 Induction of apoptosis by 1,25-D<sub>3</sub> involves disruption of mitochondrial function. *Left*. ROS production. *Right*. Mitochondrial membrane potential. (Unpublished results)

act solely as executioners to facilitate 1,25-D<sub>3</sub> mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-D<sub>3</sub>.

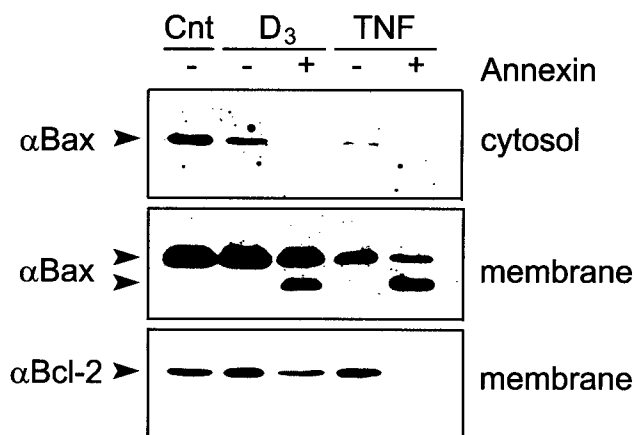


Figure 2 Subcellular distribution of Bax and Bcl-2 in fluorescence-activated cell sorted populations of viable and apoptotic cells (unpublished results).

To further support a role for the Bcl-2 family of apoptotic regulators in 1,25-D<sub>3</sub> mediated apoptosis, fluorescence-activated cell sorting of annexin V-FITC was used to obtain populations enriched in viable and apoptotic cells. Loss of cytosolic Bax and enrichment of membrane bound Bax, as well as down-regulation of Bcl-2, was observed in the apoptotic cell population generated by treatment with 1,25-D<sub>3</sub> (Figure 2).

The observation that 1,25-D<sub>3</sub> triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells suggest that 1,25-D<sub>3</sub> can activate downstream effector caspases. Since cytochrome *c* release has been associated with autoactivation of procaspase-9, 1,25-D<sub>3</sub> may activate caspase-dependent pathways via

cytochrome *c* release. However, no DEVDase activity was detected in 1,25-D<sub>3</sub> treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1,25-D<sub>3</sub>. Caspase activity assays are currently underway to determine which caspase is responsible for 1,25-D<sub>3</sub> mediated caspase dependent events.

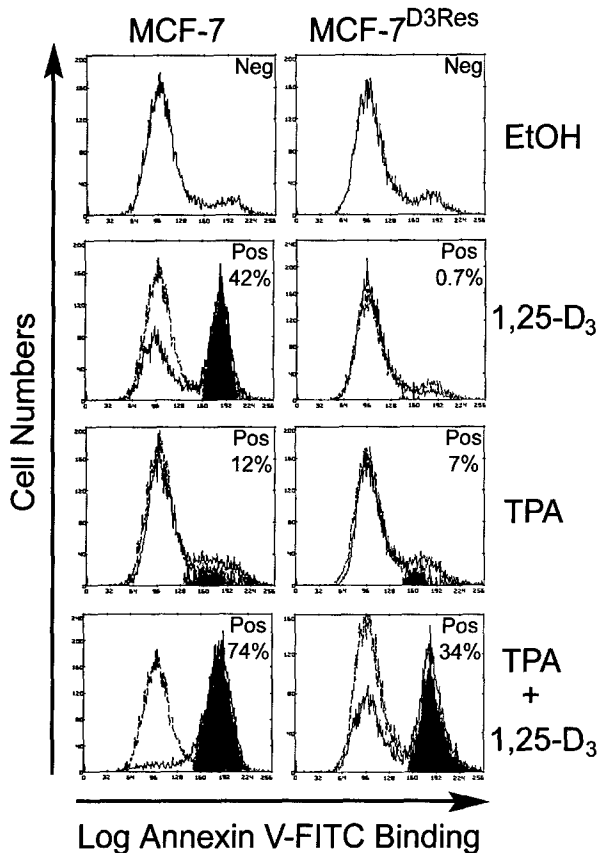


Figure 3 Phosphatidylserine exposure as determined by flow cytometric analysis of annexin V-FITC binding in MCF-7 or MCF-7<sup>D3Res</sup> cells after treatment with 1,25-D<sub>3</sub>±TPA (unpublished results).

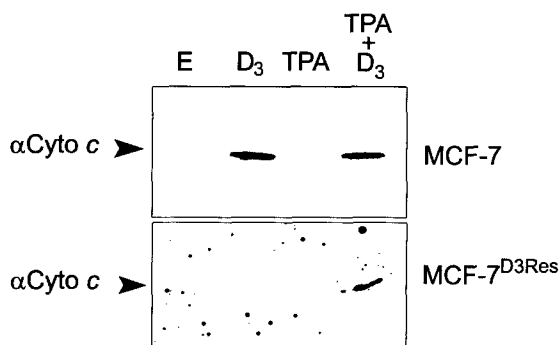


Figure 4 Redistribution of cytochrome *c* in MCF-7 or MCF-7<sup>D3Res</sup> cells after treatment with 1,25-D<sub>3</sub>±TPA (unpublished results).

The original scope of this study was to examine the role of phosphorylation pathways in vitamin D<sub>3</sub> induced apoptosis. Previous studies have shown that the VDR can be phosphorylated by protein kinase C (PKC) and casein kinase II (Hsieh *et al.*, 1993; Jurutka *et al.*, 1993; Hilliard *et al.*, 1994). Our observation that the phorbol ester TPA, a PKC activator, can amplify the effects of 1,25-D<sub>3</sub> on induction of apoptosis in MCF-7 cells, and that TPA can partially sensitize the vitamin D<sub>3</sub> resistant variant to the effects of 1,25-D<sub>3</sub> suggests an important role for phosphorylation in dictating sensitivity to vitamin D<sub>3</sub> mediated apoptosis (Narvaez and Welsh, 1997). In the second Annual Report, I demonstrated that mitochondrial disruption and caspase activity are abrogated in response to 1,25-D<sub>3</sub> treatment in the MCF-7<sup>D3Res</sup> cells. However, when the MCF-7<sup>D3Res</sup> cells are treated with 1,25-D<sub>3</sub> in the presence of TPA, activation of caspases is observed as assessed by phosphatidylserine exposure (Figure 3). This shows that the effects of 1,25-D<sub>3</sub> on mitochondrial disruption and caspase activity might be susceptible to activators of PKC.

To further expand on these findings, cytochrome *c* release and Bax translocation were examined in the parental MCF-7 and MCF-7<sup>D3Res</sup> cells in the presence of TPA±1,25-D<sub>3</sub> to determine whether PKC activators can potentiate the effects of 1,25-D<sub>3</sub> on mitochondrial activity. As demonstrated in Figure 4, the parental MCF-7 cells released cytochrome *c* in response to 1,25-D<sub>3</sub> in the presence or absence of TPA, whereas the MCF-7<sup>D3Res</sup> cells released cytochrome *c* only in the presence of both TPA and 1,25-D<sub>3</sub>. TPA on its own did not induce redistribution of cytochrome *c* to the cytosol demonstrating that it has no effect on mitochondria. This observation was confirmed by examination of the subcellular localization of cytochrome *c* by fluorescence microscopy. In addition, the subcellular localization of Bax was also examined. In Figure 5 A&B, cytochrome *c* (bottom panels) and Bax (middle panels) staining is presented beside phase contrast images (top panels) to compare cytochrome *c* and Bax localization in individual viable and apoptotic cells. In vehicle-treated control cells,



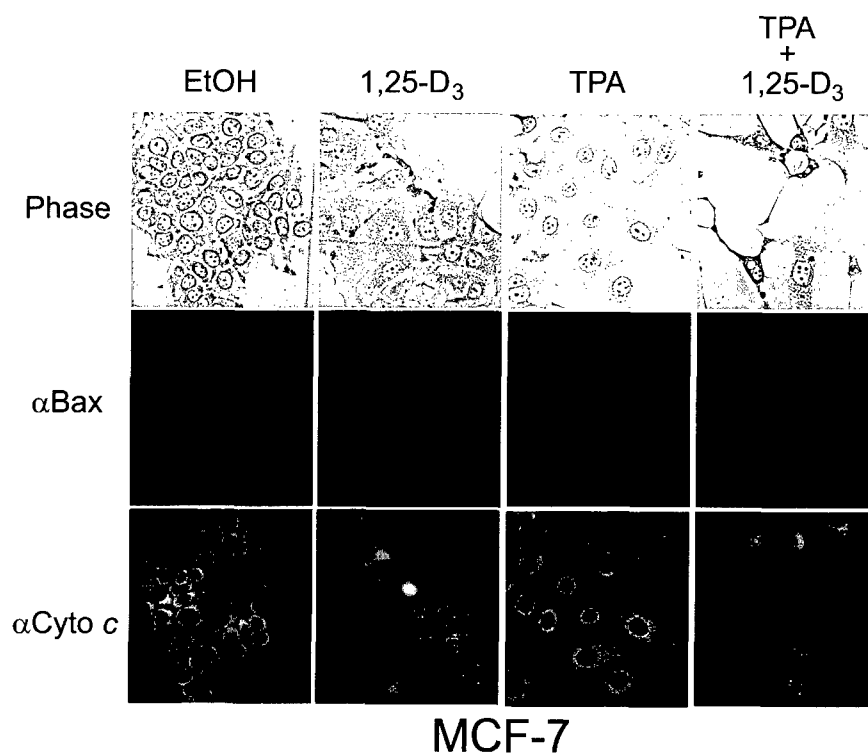
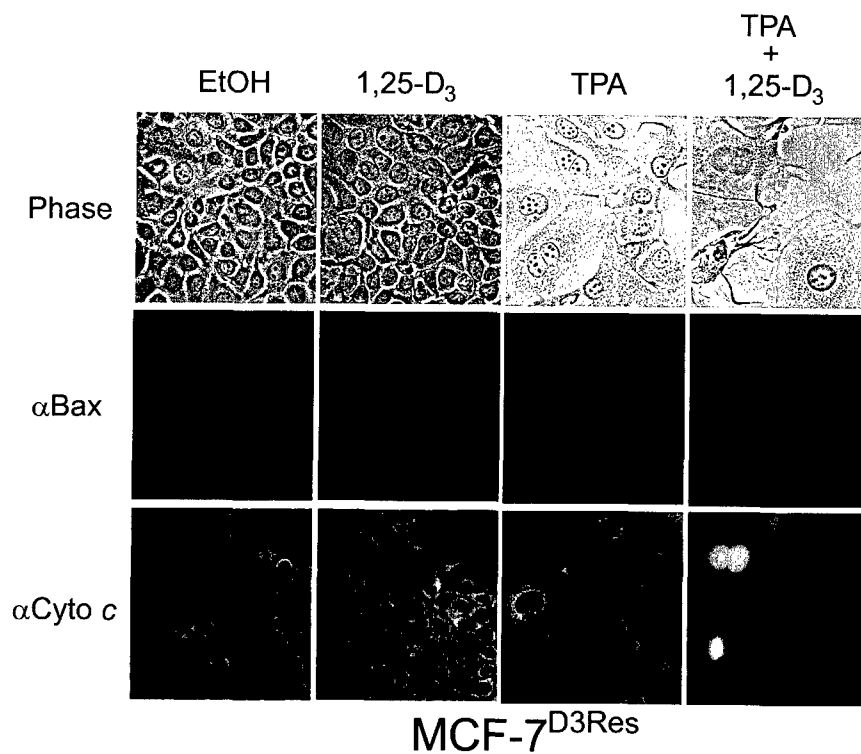
**A****B**

Figure 5 Morphology, and subcellular localization of Bax and cytochrome *c* in (A) parental MCF-7 and (B) MCF-7<sup>D3Res</sup> cells after treatment with 1,25-D<sub>3</sub>±TPA (unpublished results).

apoptotic morphology was not present, Bax staining was diffuse whereas cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization. After treatment with 1,25-D<sub>3</sub> in the presence or absence of TPA in parental MCF-7 cultures, cells identified as apoptotic by phase contrast exhibited nuclear and cytoplasmic condensation, and cytosolic vacuolization. In these apoptotic cells, diffuse cytoplasmic cytochrome *c* staining was detected throughout the cell, which obscured the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm. In contrast, Bax exhibited punctate staining in these apoptotic cells, demonstrating the translocation of Bax from the cytosol to the mitochondria. Punctate Bax staining was also observed in cells which still retained mitochondrial cytochrome *c* indicating that translocation of Bax to mitochondria always precedes cytochrome *c* release, and is one of the initiating events leading to disruption of mitochondrial activity. TPA treated MCF-7 cultures contained many giant cells with large, round nuclei and secretory granules. Bax staining remained diffuse indicating cytosolic localization whereas cytochrome *c* staining was distributed mainly in the mitochondria, thus demonstrating that TPA on its own did not induce redistribution of cytochrome *c* or Bax. The MCF-7<sup>D3Res</sup> cells displayed apoptotic morphology and redistribution of both cytochrome *c* and Bax when treated with 1,25-D<sub>3</sub> in the presence of TPA (Figure 5, B). Signals generated by 1,25-D<sub>3</sub> that induce Bax translocation to mitochondria are currently unknown. Since events upstream of Bax translocation to mitochondria in response to 1,25-D<sub>3</sub> are abrogated in the vitamin D<sub>3</sub> resistant MCF-7 variant but can be sensitized via the PKC activator TPA, comparison of early events in VDR signaling and phosphorylation in these cells will be an important subject for future studies.

The effect of TPA on VDR binding to DNA and transactivation ability has also been examined. Nuclear extracts of MCF-7 cells treated with TPA had a diminished DNA binding capacity (measured by electromobility shift analysis) compared to either control or 1,25-D<sub>3</sub> treated extracts (data shown in the first Annual Report). These data demonstrate that phosphorylation has a role in DNA binding of the VDR. Transcriptional activity of the VDR was assessed by reporter gene assay with a 24-hydroxylase luciferase construct. Basal transcriptional activity was similar in parental MCF-7 and MCF-7<sup>D3Res</sup> cell lines, however, 1,25-D<sub>3</sub> stimulated 24-hydroxylase luciferase activity was blunted in MCF-7<sup>D3Res</sup> cells

compared to parental MCF-7. In Figure 6, TPA pretreatment greatly enhanced in a dose dependent manner 1,25-D<sub>3</sub> stimulated 24-hydroxylase luciferase activity in parental MCF-7 cells. In MCF-7<sup>D3Res</sup> cells, TPA pretreatment slightly enhanced 1,25-D<sub>3</sub> stimulated 24-hydroxylase luciferase activity but the induction remained much lower than the parental MCF-7 cells. The vitamin D<sub>3</sub> resistant variant MCF-7 cells may be more sensitive to the transient nature of phosphorylation events. Effects of TPA co-treatments will be examined

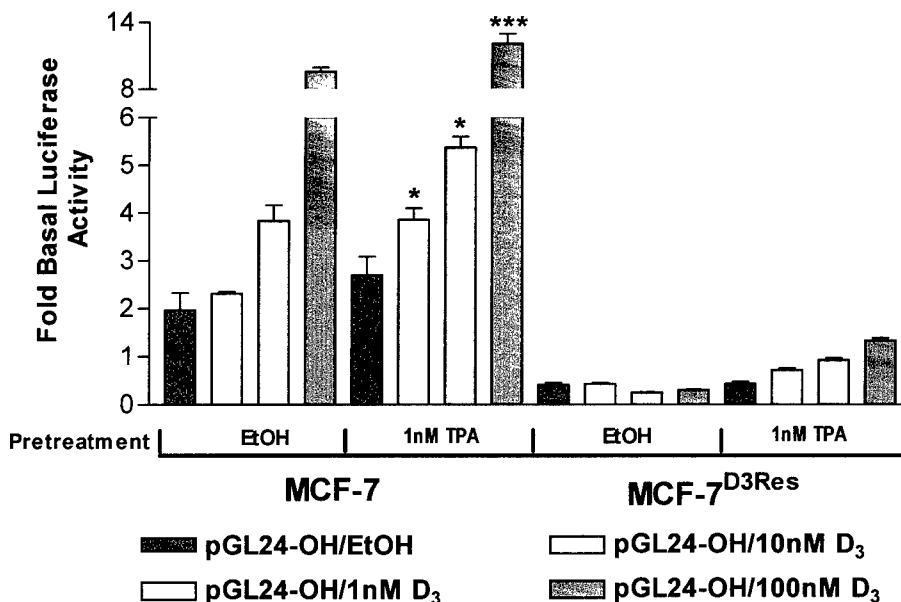


Figure 6 Effects of TPA pretreatment on transcriptional activity of the VDR using a 24-hydroxylase luciferase construct on 1,25-D<sub>3</sub> dose response in parental MCF-7 or MCF-7<sup>D3Res</sup> cells (unpublished results). \*\*\*  $p < 0.001$ ; \*  $p < 0.05$ .

next. In addition, the presence and overall concentrations of co-repressors and co-activators in the two cell lines should also be examined.

By understanding the mechanism of 1,25-D<sub>3</sub> mediated apoptosis, one can determine how TPA (and PKC pathway) interact with vitamin D signaling in potentiating apoptosis in D<sub>3</sub>-resistant MCF-7 cells.

## STATEMENT OF WORK

**Task 1:** *Compare phosphorylation state of the VDR in MCF-7 and MCF-7<sup>D3Res</sup> cells following treatment with ethanol, 1,25-D<sub>3</sub> and TPA. Conduct time courses (up to 24 hrs) and dose responses (1-100 nM).*

**Task 2:** *Conduct 2D peptide mapping and phosphoamino acid analysis to determine if distinct sites are phosphorylated after treatment with 1,25-D<sub>3</sub> or TPA and determine whether differences correlate to sensitivity to apoptosis.*

Due to technical difficulties as outlined in the first Annual Report, Tasks 1&2 were not completed.

**Task 3:** *Monitor biochemical and morphological indices of apoptosis to determine whether differences in phosphorylation patterns correlate to sensitivity to apoptosis.*

Completed.

**Task 4:** *Conduct gel shift assays to determine whether phosphorylation affects binding of the VDR to selective VDREs and whether VDR binding is altered in MCF-7 and MCF-7<sup>D3Res</sup> cells after treatment with 1,25-D<sub>3</sub> or TPA±1,25-D<sub>3</sub>.*

Partially completed. Assessment of VDR binding to selective VDREs in MCF-7<sup>D3Res</sup> cells was not completed.

**Task 5:** *Subclone multiple copies of chosen VDREs into Luciferase reporter plasmid.*

Completed.

**Task 6:** *Conduct transient transfections and Luciferase assays on MCF-7 and MCF-7<sup>D3Res</sup> cells treated with 1,25-D<sub>3</sub> or TPA±1,25-D<sub>3</sub> to determine if differences in VDR phosphorylation and sequence specific DNA binding translate to alterations in VDR mediated transactivation in the whole cell system.*

Completed.

## KEY RESEARCH ACCOMPLISHMENTS

### First Year

- ✓ First observation implicating a role for mitochondrial events in 1,25-D<sub>3</sub> mediated apoptosis.
- ✓ **Caspase independent events** involved in 1,25-D<sub>3</sub> mediated apoptosis.
  - Translocation of Bax
  - Release of cytochrome *c*
  - Production of reactive oxygen species
- ✓ **Caspase dependent events** involved in 1,25-D<sub>3</sub> mediated apoptosis.
  - PARP cleavage
  - External display of phosphatidylserine
  - DNA fragmentation
- ✓ The observation that 1,25-D<sub>3</sub> mediated cell death is caspase independent.
- ✓ Techniques acquired so far in the course of this study
  - Flow Cytometry
  - Immunoprecipitation
  - Electromobility Shift Assay

### Second Year

- ✓ Time course studies of cytochrome *c* release, ROS production, and phosphatidylserine exposure suggests that mitochondrial disruption occurs prior to activation of downstream caspases.
- ✓ The observation that TPA potentiates the effects of 1,25-D<sub>3</sub> on MCF-7<sup>D3Res</sup> cells by activating downstream caspases, thus indicating that the effects of 1,25-D<sub>3</sub> on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.
- ✓ The observation that 1,25-D<sub>3</sub> stimulated 24-hydroxylase luciferase activity is blunted in MCF-7<sup>D3Res</sup> cells compared to parental MCF-7.

### Third Year

- ✓ Fluorescence activated cell sorting of annexin V-FITC to obtain populations enriched in viable and apoptotic cells.
- ✓ Strong evidence that demonstrates involvement of Bcl-2 family of apoptotic regulators in 1,25-D<sub>3</sub> mediated apoptosis.
  - Western blot analysis demonstrated loss of cytosolic Bax, enrichment of membrane bound Bax, as well as down-regulation of Bcl-2 in the apoptotic cell population generated by treatment with 1,25-D<sub>3</sub>.
  - Immunocytofluorescence evidence showed redistribution of Bax from cytosol to mitochondria after treatment with 1,25-D<sub>3</sub>.
- ✓ The observation that translocation of Bax to mitochondria always precedes cytochrome *c* release, and is one of the initiating events leading to disruption of mitochondrial activity.

### **KEY RESEARCH ACCOMPLISHMENTS (cont'd)**

- ✓ The observation that TPA on its own did not alter mitochondrial function but was able to augment the effects of 1,25-D<sub>3</sub> on mitochondria and caspase activity.
- ✓ The observation that TPA pretreatment enhanced 1,25-D<sub>3</sub> stimulated 24-hydroxylase luciferase activity, although induction remained lower in the MCF-7<sup>D3Res</sup> cells compared to the parental cell line.

## REPORTABLE OUTCOMES

### First Year

- ✓ Poster presentation at the 1999 Keystone Symposia “**Apoptosis and Programmed Cell Death**” at Breckenridge, CO, April 6-11, 1999. Abstract title: “Caspase-independent apoptosis by vitamin D treatment in MCF-7 breast cancer cells.”
- ✓ Awarded a Travel Award for the poster presentation at the 1999 Keystone Symposium.

### Second Year

- ✓ Narvaez C.J., Waterfall T., Welsh J. (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. In Norman, AW, Bouillon, R, and Thomasett, M (eds), *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects*. University of California, Riverside, pp. 383-386
- ✓ Abstract promoted to a 10 min presentation at the **Eleventh Workshop on Vitamin D** in Nashville, TN, May 27-June 1, 2000. Abstract Title: “Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells.”
- ✓ Poster presented at the *Era of Hope* **Department of Defense Breast Cancer Research Program Meeting** in Atlanta, GA, June 8-11, 2000. Abstract Title: “Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells.”

### Third Year

- ✓ Narvaez C.J. and Welsh J. (2001) Role of mitochondria and caspases in vitamin D mediated apoptosis of MCF-7 breast cancer cells. *J Biol Chem* **276**:9101-9107
- ✓ Narvaez C.J., Zinser G., and Welsh J. (2001) Functions of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> in mammary gland: from normal development to breast cancer. *Steroids* **66**:301-308
- ✓ Poster presented at the **83<sup>rd</sup> Annual Meeting of the Endocrine Society** in Denver, CO, June, 2001. Abstract Title: “Disruption of mitochondrial activity and induction of apoptosis by 1,25-dihydroxyvitamin D<sub>3</sub> in MCF-7 breast cancer cells.”

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1. Narvaez, C. J., Waterfall, T., and Welsh, J. (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. In Norman, A. W., Bouillon, R., and Thomasett, M. (eds.), *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects*. University of California, Riverside, pp. 383-386
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3. Narvaez, C. J. and Welsh, J. (2001) Role of mitochondria and caspases in vitamin D mediated apoptosis of MCF-7 breast cancer cells. *J. Biol. Chem.* 276:9101-9107

### ABSTRACTS:

1. Narvaez, C. J. and Welsh J. (1999) Caspase-independent apoptosis by vitamin D treatment in MCF-7 breast cancer cells. Presented at the 1999 Keystone Symposium on "*Apoptosis and Programmed Cell Death*" in Breckenridge, CO, April, 1999
2. Narvaez, C. J. and Welsh, J. (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. Abstract promoted to a 10 min presentation at the Eleventh Workshop on Vitamin D in Nashville, TN, June 2000
3. Narvaez, C. J. and Welsh, J. (2000) Role of mitochondria and caspases in vitamin D mediated apoptosis in MCF-7 breast cancer cells. Presented at the *Era of Hope* Department of Defense Breast Cancer Research Program in Atlanta, GA, June, 2000
4. Narvaez, C. J., Brown, M., and Welsh, J. (2001) Disruption of mitochondrial activity and induction of apoptosis by 1,25-dihydroxyvitamin D<sub>3</sub> in MCF-7 breast cancer cells. Presented at the 83<sup>rd</sup> Annual Meeting of the Endocrine Society in Denver, CO, June, 2001

## CONCLUSION

1,25-D<sub>3</sub> mediates apoptosis of MCF-7 cells through mitochondrial signaling which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-D<sub>3</sub> mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-D<sub>3</sub>. Most significantly, the observation that TPA can partially sensitize the vitamin D<sub>3</sub> resistant variant to the effects of 1,25-D<sub>3</sub> suggests an important role for phosphorylation in dictating sensitivity to vitamin D<sub>3</sub> mediated apoptosis. This indicates that the effects of 1,25-D<sub>3</sub> on mitochondrial disruption and caspase activity might be sensitized through activators of PKC.

By understanding the effects of 1,25-D<sub>3</sub> on mitochondria and caspase activity, one can determine how TPA (and the PKC pathway) interact with vitamin D signaling in potentiating apoptosis in D<sub>3</sub>-resistant MCF-7 cells. This project has important implications for breast cancer since a breast cancer cell that is resistant to the apoptosis-inducing effects of 1,25-D<sub>3</sub> might be sensitized through activators of PKC. This basic knowledge could lead to new therapeutics for treatment of certain forms of breast cancer.



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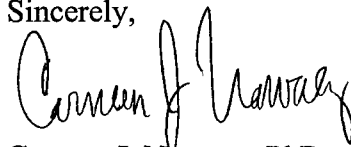
Commander  
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Dear Sir or Madam:

Re: Annual Report for "The Role of VDR Phosphorylation in Vitamin D Induced Apoptosis"  
DAMD17-97-1-7183

This report contains unpublished data. All the figures contain statements indicating that the data is unpublished. Some of the data has been presented in poster format at a conference, but the data has not been submitted to a peer reviewed journal for publication yet.

Sincerely,

A handwritten signature in cursive script, appearing to read "Carmen J. Narvaez".

Carmen J. Narvaez, PhD

## Role of Mitochondria and Caspases in Vitamin D-mediated Apoptosis of MCF-7 Breast Cancer Cells\*

Received for publication, July 31, 2000, and in revised form, October 2, 2000  
Published, JBC Papers in Press, October 26, 2000, DOI 10.1074/jbc.M006876200

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Vitamin D<sub>3</sub> compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis in breast cancer cells (MCF-7) *in vitro*. The effect of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on MCF-7 cells was compared with that of tumor necrosis factor  $\alpha$ , which induces apoptosis via a caspase-dependent pathway. Our major findings are that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> induces apoptosis in MCF-7 cells by disruption of mitochondrial function, which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species. Moreover, we show that Bax translocation and mitochondrial disruption do not occur after 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment of a MCF-7 cell clone selected for resistance to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis. These mitochondrial effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> do not require caspase activation, since they are not blocked by the cell-permeable caspase inhibitor z-Val-Ala-Asp-fluoromethylketone. Although caspase inhibition blocks 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated events downstream of mitochondria such as poly(ADP-ribose) polymerase cleavage, external display of phosphatidylserine, and DNA fragmentation, MCF-7 cells still execute apoptosis in the presence of z-Val-Ala-Asp-fluoromethylketone, indicating that the commitment to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated cell death is caspase-independent.

1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>),<sup>1</sup> the active form of vitamin D<sub>3</sub>, acts through the nuclear vitamin D<sub>3</sub> receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo* (1). A variety of synthetic

vitamin D<sub>3</sub> analogs that induce mammary tumor regression in animals are now undergoing clinical trials in human patients (2, 3). Our laboratory has shown that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in breast cancer cells (MCF-7) (4, 5); however, the precise mechanism by which 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and the VDR mediate apoptosis is poorly understood.

To characterize the mechanisms of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis in breast cancer cells, we compared specific intracellular events in MCF-7 cells after treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). TNF $\alpha$  was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by a well defined pathway triggered by tumor necrosis factor receptor 1 (TNFR1), a cell surface death receptor. Death receptors contain homologous cytoplasmic regions termed "death domains," which transmit apoptotic signals through recruitment of adaptor molecules that activate caspases, a family of cysteine proteases involved in cell disassembly. The best characterized death receptors (Fas, TNFR1) use Fas-associated death domain and TNFR1-associated death domain adaptors to recruit and activate caspase-8 (6). Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, including poly(ADP-ribose) polymerase (PARP), and lead to external display of phosphatidylserine (PS), DNA fragmentation, and cellular condensation (7).

Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of the outer mitochondrial membrane, decreased transmembrane potential, release of cytochrome *c* and apoptosis-inducing factor, and production of reactive oxygen species (ROS) (8, 9). Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X<sub>L</sub>, can block these mitochondrial events, whereas pro-apoptotic Bcl-2 family members, including Bax, can trigger these changes. For example, apoptotic signals induce conformational changes in Bax, which lead to exposure of the pro-apoptotic BH3 domain, and translocation to the mitochondria (10). The effects of pro-apoptotic Bcl-2 family members are achieved by both caspase-dependent and caspase-independent mechanisms (11, 12).

Although the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase-dependent pathways. To probe the mechanisms whereby vitamin D<sub>3</sub> signaling modulates apoptosis in MCF-7 cells, we studied the effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on mitochondrial function and caspase activity using a cell-permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk). In addition, the effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and TNF $\alpha$  on a vitamin D<sub>3</sub>-resistant variant of MCF-7 cells (MCF-7<sup>D3Res</sup> cells) were examined to identify events that contribute to vitamin D<sub>3</sub> resistance (13, 14). The MCF-7<sup>D3Res</sup> cells do not undergo cell cycle arrest or apoptosis in response to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>; however,

\* This work was supported by Army Breast Cancer Research Program Grant DAMD17-97-1-7183 and NCI National Institutes of Health Grant CA69700. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; z, benzyloxycarbonyl; Ac, acetyl; VAD, Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; IETD, Ile-Glu-Thr-Asp; fmk, fluoromethylketone; CHO, aldehyde; AFC, 7-amino-4-trifluoromethylcoumarin; DEVDase, DEVD-cleavage specific caspase; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TNFR1, tumor necrosis factor receptor 1; VDR, vitamin D<sub>3</sub> receptor; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; PS, phosphatidylserine; PI, propidium iodide; PBS, phosphate-buffered saline; TMRE, tetramethylrhodamine ethyl ester; HE, hydroethidine; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin.

these cells retain sensitivity to other inducers of apoptosis such as TNF $\alpha$  and anti-estrogens (13).

Our results indicate that, although caspase inhibition can block some of the late stages of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis in MCF-7 cells, the commitment to cell death is caspase-independent. These data implicate Bax distribution and mitochondrial disruption as critical caspase-independent events in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis of breast cancer cells.

#### EXPERIMENTAL PROCEDURES

**Cells and Cell Culture**—MCF-7 cells (originally obtained from ATCC) were used to generate the vitamin D<sub>3</sub>-resistant variant (MCF-7<sup>D3Res</sup>) that has been described previously (13). Both cell lines were cultured in  $\alpha$ -minimal essential medium (Life Technologies, Inc.) containing 25 mM HEPES and 5% fetal bovine serum (Life Technologies, Inc.). Cells were routinely plated at 5000 cells/cm<sup>2</sup> and passaged every 3–4 days. Stock cultures of MCF-7<sup>D3Res</sup> cells were routinely grown in medium containing 100 nM 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (kindly provided by LEO Pharmaceuticals, Ballerup, Denmark). For experiments, MCF-7 and MCF-7<sup>D3Res</sup> cells were plated in  $\alpha$ -minimal essential medium containing 5% fetal bovine serum plus antibiotics, and treated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or ethanol vehicle 2 days after plating. Parallel cultures were treated with TNF $\alpha$  (Sigma) 2–3 days before scheduled harvest of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-treated dishes. Caspase inhibitors, zVAD.fmk or zDEVD.fmk (Enzyme Systems Products, Livermore, CA) were added at the same time (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or TNF $\alpha$ ) or 2 days after (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) initial treatment. For cell growth assays, cells were seeded at 1000 cells/well in 24-well plates, treated for the indicated times, and analyzed by crystal violet assay. Briefly, cells were fixed with 1% glutaraldehyde for 15 min, incubated with 0.1% crystal violet (Fisher Scientific, Pittsburgh, PA) for 30 min, destained with H<sub>2</sub>O, and solubilized with 0.2% Triton X-100. Absorbance at 562 nm (minus background at 630 nm) was determined on a microtiter plate reader.

**Clonogenic Assay**—MCF-7 cells were incubated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> for 6 days or TNF $\alpha$  for 1 day in the presence or absence of 25  $\mu$ M zVAD.fmk. For 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment, medium was replaced every 2 days. After treatments, cells were trypsinized, media and washes were pooled, and cells were pelleted by centrifugation and resuspended in fresh medium. The cells were seeded in 96-well plates at 5, 50, 500, and 2500 cells/well in 24 replicates. After 14 days, the cells were fixed and stained with crystal violet as described above, and clonogenic potential was estimated by counting positive wells (15).

**Subcellular Fractionation**—Cells were trypsinized, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500  $\times$  g for 3 min at 4  $^{\circ}$ C. Pellets were resuspended with 3 volumes of Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 10 mM benzamide, 1 mM dithiothreitol, 250 mM sucrose, plus protease and phosphatase inhibitors), lysed with a Dounce homogenizer, and fractionated by differential centrifugation (16). Briefly, homogenates were centrifuged twice at 500  $\times$  g for 5 min at 4  $^{\circ}$ C, and the nuclear pellet was resuspended in Buffer A, sonicated 2  $\times$  10 s, and stored at -80  $^{\circ}$ C in multiple aliquots. The supernatants were combined and further centrifuged at 10,000  $\times$  g for 30 min at 4  $^{\circ}$ C, and the resultant mitochondrial pellets were resuspended in Buffer A, sonicated, and stored at -80  $^{\circ}$ C in multiple aliquots. The supernatant from the 10,000  $\times$  g spin was further centrifuged at 100,000  $\times$  g for 1 h at 4  $^{\circ}$ C. The resulting supernatant was designated S100 (containing cytosol) and stored at -80  $^{\circ}$ C in multiple aliquots. Protein concentrations were determined by the Micro BCA protein assay (Pierce).

**Immunoblot Analysis**—Subcellular fractions isolated as described above were solubilized in Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Proteins derived from mitochondria and/or S100 extracts were immunoblotted with Bax rabbit polyclonal (13666E; PharMingen, San Diego, CA) or cytochrome c mouse monoclonal antibodies (7H8.2C12; PharMingen) diluted 1:500 or 1:250, respectively, in PBS plus 5% skim milk. S100 extracts were also probed with cytochrome oxidase subunit II mouse monoclonal antibody (clone 12C4-F12; Molecular Probes, Eugene, OR) to exclude mitochondrial contamination. Nuclear extracts were probed with PARP mouse monoclonal antibody (C2.10, Enzyme Systems Products) diluted 1:1000. Specific antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) diluted 1:5000 in PBS, 5% skim milk, 0.1% Tween 20 and autoradiography with enhanced chemiluminescence (Pierce).

**Immunocytochemistry**—MCF-7 cells grown on Lab-Tek II chamber slides (Fisher Scientific) were treated with ethanol vehicle, 100 nM

1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, or 2.5 ng/ml TNF $\alpha$  for 96 h (ethanol, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) or 48 h (TNF $\alpha$ ) in the presence or absence of 25  $\mu$ M zVAD.fmk. The cells were fixed in 4% formaldehyde in PBS for 5 min at room temperature, permeabilized in methanol at -20  $^{\circ}$ C for 5 min, and blocked overnight with PBS plus 1% BSA containing 0.02% sodium azide. The slides were then incubated with cytochrome c mouse monoclonal antibody (6H2.B4; PharMingen), diluted 1:100 in blocking buffer, for 3 h at 37  $^{\circ}$ C in a humidified chamber. Slides were washed three times for 5 min each time with PBS, followed by incubation for 1 h at room temperature with anti-mouse secondary antibody conjugated to Alexa-488 (a photostable dye with spectral properties similar to fluorescein; Molecular Probes) diluted 1:50 in blocking buffer. Slides were washed three times for 5 min with PBS, incubated for 15 min at room temperature with 1  $\mu$ g/ml Hoechst 33258 (Sigma), washed five times for 5 min with PBS, rinsed with distilled H<sub>2</sub>O, and coverslips were applied with an antifade reagent. Fluorescence was detected using an Olympus AX70 microscope equipped with a Spot RT digital camera.

**Flow Cytometry**—For analysis of mitochondrial membrane potential and reactive oxygen species, cells harvested by trypsinization were pooled with media plus washes and pelleted by centrifugation. Cell suspensions (1  $\times$  10<sup>6</sup> cells) were incubated with 1  $\mu$ M tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) in PBS containing 130 mM KCl to abolish the plasma membrane potential. After incubation for 10 min at 37  $^{\circ}$ C, cells were washed once in PBS and then analyzed for TMRE red fluorescence by flow cytometry. Live cells rapidly and reversibly take up TMRE, and accumulation of the dye in mitochondria has been shown to be potential driven (17). For analysis of ROS, cell suspensions (5  $\times$  10<sup>5</sup> cells) were incubated with 4  $\mu$ M hydroethidine (HE, Molecular Probes) in PBS for 15 min at 37  $^{\circ}$ C, and conversion of HE to ethidium by superoxide anion was analyzed by flow cytometry.

For analysis of DNA fragmentation, MCF-7 cells were harvested by trypsinization, collected by centrifugation, fixed in 2% formaldehyde in PBS, and permeabilized in 70% EtOH at -20  $^{\circ}$ C. DNA strand breaks in cells undergoing apoptosis were indirectly labeled with bromodeoxyuridine by terminal transferase (Roche Molecular Biochemicals) and detected by FITC-conjugated monoclonal antibody to bromodeoxyuridine using the APO-BRDU kit according to manufacturer's protocol (Phoenix Flow Systems, San Diego, CA). Cells were counterstained with 5  $\mu$ g/ml propidium iodide (PI; Sigma) containing RNase A (Roche Molecular Biochemicals) for detection of total DNA, and two-color analysis of DNA strand breaks and cell cycle was achieved by flow cytometry.

For detection of PS externalization, 1  $\times$  10<sup>6</sup> cells were incubated in the presence of 10  $\mu$ g/ml annexin V-FITC (BioWhittaker, Walkersville, MD) and 5  $\mu$ g/ml PI in binding buffer (10 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>) for 15 min at 37  $^{\circ}$ C. Cells were washed twice in binding buffer, fixed in 2% formaldehyde in PBS for 15 min on ice, and then washed two more times in PBS plus 0.2% BSA. Pellets were resuspended in PBS plus 0.2% BSA and analyzed by flow cytometry. There were less than 1% PI<sup>+</sup> cells in the population, and they were therefore excluded from analysis.

All flow cytometric analyses were performed on an Epics XL Flow Cytometer (Coulter Corp., Miami, FL) equipped with an argon laser. TMRE and HE were analyzed on FL3 using a 620-nm band pass filter. For DNA fragmentation analysis, FITC was analyzed on FL1 using a 520-nm band pass filter and PI was analyzed on FL3 with no color compensation. For PS externalization, annexin V-FITC was analyzed on FL1 and PI was analyzed on FL2 (580-nm band pass filter) using software color compensation. Data was modeled with the Multiplus AV software (Phoenix Flow Systems).

**Caspase Activity Assay**—Caspase activity was analyzed with the ApoAlert CPP32/caspase-3 assay kit according to manufacturer's protocol (CLONTECH, Palo Alto, CA). Briefly, after harvesting by trypsinization, 2  $\times$  10<sup>6</sup> cells were pelleted and stored at -20  $^{\circ}$ C. For analysis, cell pellets were lysed, re-pelleted to remove cell debris, and supernatants were incubated with 50  $\mu$ M DEVD-AFC for 1 h at 37  $^{\circ}$ C. The samples were analyzed using a fluorescence spectrophotometer with excitation = 380 nm and emission = 508 nm.

**Statistical Evaluation**—Data are expressed as mean  $\pm$  S.E. One-way analysis of variance was used to assess statistical significance between means. Differences between means were considered significant if *p* values less than 0.05 were obtained with the Bonferroni method using GraphPad Instat software (GraphPad Software, San Diego, CA).

#### RESULTS

**Disruption of Mitochondrial Function, as Determined by Subcellular Localization of Bax and Cytochrome c, and ROS Generation, by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>**—To identify specific intracellular events

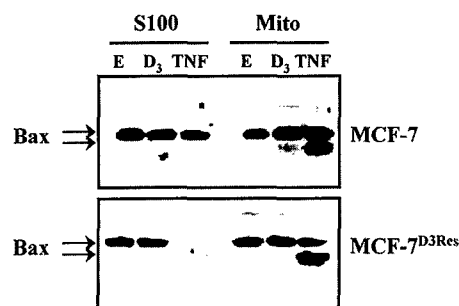


FIG. 1. Subcellular distribution of Bax after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in parental MCF-7 or MCF-7<sup>D3Res</sup> cells. MCF-7 or MCF-7<sup>D3Res</sup> cells were plated at a density of  $2 \times 10^5$  cells/150-mm dish. Two days after plating, cells were treated with vehicle control (ethanol) or 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  for 96 h, or with 2.5 ng/ml  $\text{TNF}\alpha$  for 48 h. Mitochondria and S100 isolated as described under "Experimental Procedures" were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal antibody to Bax. The results are representative of at least three independent experiments.

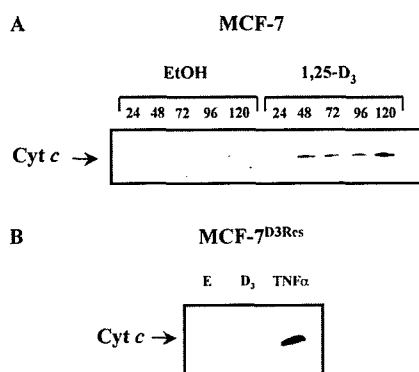


FIG. 2. Cytosolic localization of cytochrome *c* after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$ . **A**, time course of cytosolic cytochrome *c* after treatment with vehicle control (ethanol) or 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  in MCF-7 cells. Cells were plated at a density of  $1 \times 10^5$  cells/150-mm dish. Two days after plating, the cells were treated with ethanol or  $1\alpha,25(\text{OH})_2\text{D}_3$  and re-fed 2 days later. S100 fractions prepared at the indicated time points as described under "Experimental Procedures" were separated on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with cytochrome *c* (7H8.2C12) antibody. **B**, cytosolic cytochrome *c* in MCF-7<sup>D3Res</sup> cells. Cells were plated and treated with ethanol or  $1\alpha,25(\text{OH})_2\text{D}_3$  as described above, and 2.5 ng/ml  $\text{TNF}\alpha$  was added 3 days before harvest. All the dishes were harvested on day 5 of treatment, and S100 fractions were prepared and immunoblotted as described above. The results are representative of at least three independent experiments.

involved in  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated apoptosis, we examined the signaling pathway downstream of the VDR in MCF-7 cells. Since disruption of mitochondrial function is a primary event in apoptosis that can be triggered by translocation of Bax to mitochondrial outer membrane, we first examined the subcellular distribution of Bax after  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment of MCF-7 cells. As demonstrated in Fig. 1, Bax redistribution from the cytosolic to the mitochondrial fraction occurred after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in MCF-7 cells (Fig. 1, top). Not only was Bax translocated to mitochondria, but both  $1\alpha,25(\text{OH})_2\text{D}_3$ - and  $\text{TNF}\alpha$ -treated cells exhibited cleavage of Bax from the intact 21-kDa protein to an 18-kDa fragment, an observation that is consistent with reports of Bax cleavage during drug-induced apoptosis (18). In both  $1\alpha,25(\text{OH})_2\text{D}_3$ - and  $\text{TNF}\alpha$ -treated cells, the Bax cleavage product was detected in mitochondrial, but not cytosolic, fractions, and others have proposed that Bax cleavage enhances homodimerization and its pro-apoptotic properties (19). These are the first data to implicate translocation and cleavage of Bax during  $1\alpha,25(\text{OH})_2\text{D}_3$ -induced apoptosis. To determine the relationship between Bax translocation and sensitivity to

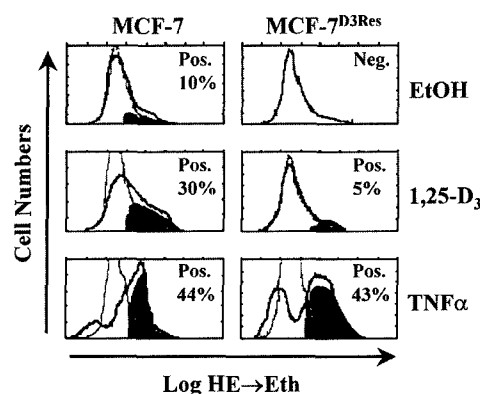


FIG. 3. ROS production after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in parental MCF-7 or MCF-7<sup>D3Res</sup> cells. Cells were plated at a density of  $1 \times 10^5$  cells/150-mm dish. Two days after plating, the cells were treated with vehicle control (ethanol), 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ , or media only, re-fed 2 days later, and 2.5 ng/ml  $\text{TNF}\alpha$  was added to dishes containing media only. All dishes were harvested on day 5 when ROS generation was assessed by flow cytometry as described under "Experimental Procedures." Data are expressed as the percentage of cells positive for ROS after negative subtraction of data derived from vehicle-treated MCF-7<sup>D3Res</sup> cells. The results are representative of at least three independent experiments.

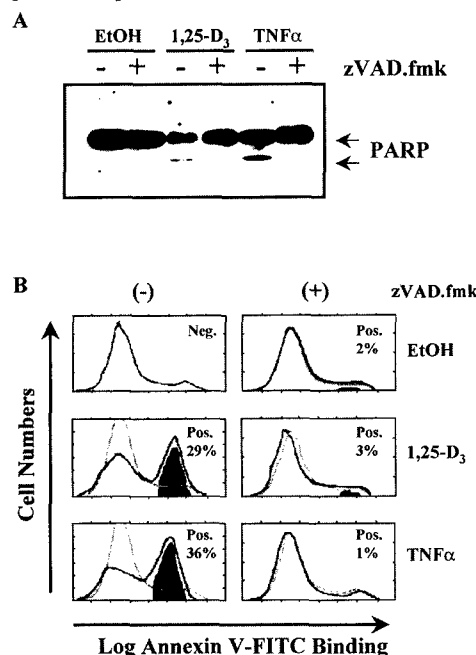
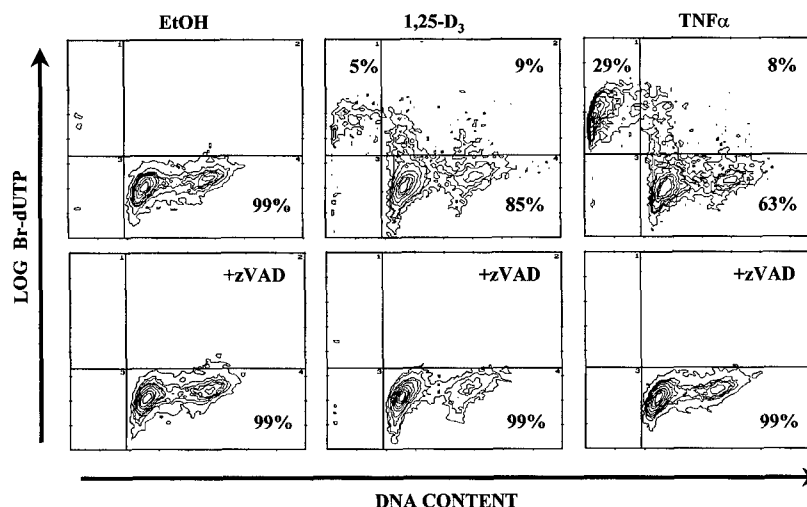


FIG. 4. Effects of caspase inhibitor on PARP cleavage and PS exposure after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in MCF-7 cells. **A**, PARP cleavage. Cells were plated and treated as described in Fig. 3 in the presence or absence of 25  $\mu\text{M}$  zVAD.fmk. All the dishes were harvested on day 5. Nuclear extracts prepared as described under "Experimental Procedures" were separated on SDS-PAGE and immunoblotted with mouse monoclonal antibody to PARP. **B**, PS externalization. Cells plated and treated as described above were incubated with annexin V-FITC and PI as described under "Experimental Procedures." Data are expressed as the percentage of annexin V-FITC-positive cells after negative subtraction of data generated with vehicle treated cells. The results are representative of at least three independent experiments.

$1\alpha,25(\text{OH})_2\text{D}_3$ -induced apoptosis, we examined the subcellular distribution of Bax in a vitamin D<sub>3</sub>-resistant variant of MCF-7 cells, which does not undergo apoptosis after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  but retains sensitivity to other triggers, including  $\text{TNF}\alpha$ .<sup>2</sup> In the MCF-7<sup>D3Res</sup> cells,  $1\alpha,25(\text{OH})_2\text{D}_3$  did not induce translocation or cleavage of Bax (Fig. 1, bottom). However, in

<sup>2</sup> C. J. Narvaez and J. Welsh, unpublished data.

FIG. 5. Effect of caspase inhibitor on DNA fragmentation after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in MCF-7 cells. Cells were plated and treated as described in Fig. 4, and DNA fragmentation was determined by flow cytometry as described under "Experimental Procedures." The results are representative of at least three independent experiments.



these cells, Bax translocation and cleavage was triggered by  $\text{TNF}\alpha$ , indicating that Bax functions appropriately in MCF-7<sup>D3Res</sup> cells during apoptosis induced by agents other than  $1\alpha,25(\text{OH})_2\text{D}_3$ . The inability of Bax to redistribute to mitochondria in response to  $1\alpha,25(\text{OH})_2\text{D}_3$  in the vitamin D<sub>3</sub>-resistant variant suggests that Bax translocation may be a critical initiating event in  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated apoptosis of MCF-7 cells.

Translocation of Bax to mitochondria has been associated with release of cytochrome c, an event that is considered a commitment point for activation of apoptosis. As expected for viable cultures, no cytochrome c was detected in cytosolic fractions from MCF-7 cells treated with ethanol vehicle for up to 120 h (Fig. 2A). In contrast, redistribution of cytochrome c from mitochondria to cytosol was detected within 48 h of  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment in MCF-7 cells, before any morphological apoptotic features were detected. The absence of cytochrome oxidase in cytosolic fractions confirmed that extracts were free of mitochondrial contamination (data not shown). In MCF-7<sup>D3Res</sup> cells,  $1\alpha,25(\text{OH})_2\text{D}_3$  did not trigger release of cytochrome c; however, cytochrome c was detected in cytosolic fractions after  $\text{TNF}\alpha$  treatment of both MCF-7 and MCF-7<sup>D3Res</sup> cell lines (Fig. 2B; see also Fig. 7).

Long term exclusion of cytochrome c from the electron transport chain can lead to impairment of proton flow and generation of ROS due to incomplete reduction of molecular oxygen. Hence, mitochondrial generation of ROS in response to  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $\text{TNF}\alpha$  was examined by flow cytometry. Production of superoxide anion was indirectly assessed as oxidation of hydroethidine to ethidium, which fluoresces red upon DNA intercalation. As presented in Fig. 3, ROS production was enhanced by  $1\alpha,25(\text{OH})_2\text{D}_3$  in MCF-7, but not MCF-7<sup>D3Res</sup> cells, whereas  $\text{TNF}\alpha$  increased ROS production comparably in both cell lines. Time-course studies have demonstrated that ROS production is enhanced within 72 h of  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment in MCF-7 cells (data not shown).

**$1\alpha,25(\text{OH})_2\text{D}_3$  Mediates PARP Cleavage, PS Externalization, and DNA Fragmentation in a Caspase-dependent Manner**—Cytochrome c released into the cytosol is thought to trigger caspase activation downstream of mitochondria through binding to Apaf-1 and autoactivation of procaspase-9. Activated caspase-9 can activate additional effector caspases responsible for cell disassembly and events such as PS externalization, PARP cleavage, and DNA fragmentation. To determine the involvement of caspase-dependent proteolysis in  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated apoptosis, we examined whether a broad spectrum cell-permeable caspase inhibitor (zVAD.fmk) could abrogate the effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  in MCF-7 cells.

Proteolytic activity associated with caspase activation was analyzed by three distinct methods: cleavage of an endogenous caspase substrate (PARP), and flow cytometric analysis of PS exposure and DNA fragmentation, which others have shown are provoked by caspases (7). As demonstrated in Fig. 4A, PARP was cleaved after treatment of MCF-7 cells with either  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$ , and in both cases, cleavage was blocked by zVAD.fmk. Furthermore, both  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $\text{TNF}\alpha$  induced PS externalization, which was also completely blocked by zVAD.fmk (Fig. 4B). Finally, the effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  and  $\text{TNF}\alpha$  on DNA fragmentation was assessed as terminal transferase-mediated incorporation of bromodeoxyuridine, detected by FITC-conjugated anti-bromodeoxyuridine antibody by flow cytometry (Fig. 5).  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment of MCF-7 cells induced DNA fragmentation primarily in the G<sub>1</sub> phase of the cell cycle, with only a small population of cells (5%) accumulating in sub-G<sub>1</sub>.  $\text{TNF}\alpha$  treatment induced extensive DNA fragmentation in the G<sub>1</sub> phase of the cell cycle, with accumulation of 29% of the population in sub-G<sub>1</sub>. Despite the differences in the magnitude and profiles of DNA fragmentation between  $1\alpha,25(\text{OH})_2\text{D}_3$ - and  $\text{TNF}\alpha$ -treated cells, zVAD.fmk completely blocked DNA fragmentation induced by both agents.

**Caspase Activity Induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  Is Not DEVDase**—DEVDase cleavage activity was measured with the fluorogenic substrate DEVD-AFC, which can detect activity of caspases-3 and -7. Since MCF-7 cells do not express functional caspase-3 (data not shown, Ref. 20), any DEVDase activity detected in these cells would most likely correspond to caspase-7. For these experiments, MCF-7 cells were pretreated with  $1\alpha,25(\text{OH})_2\text{D}_3$  for 2 days before cytosolic extracts were collected for DEVDase activity assays at the indicated time points. As demonstrated in Fig. 6,  $\text{TNF}\alpha$ , but not  $1\alpha,25(\text{OH})_2\text{D}_3$ , induced DEVDase cleavage activity in MCF-7 cells. Even with extended treatment times in additional studies, no DEVDase cleavage activity could be detected after  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment of MCF-7 cells (data not shown). This observation indicates that other known or as yet unidentified effector caspases may be activated by  $1\alpha,25(\text{OH})_2\text{D}_3$ , which mediate PARP cleavage, PS exposure, or DNA fragmentation.

**Caspase Inhibition Does Not Block  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated Cytochrome c Release, Mitochondrial Dysfunction, or Cell Death**—Since zVAD.fmk blocked  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated caspase-dependent events downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome c release and mitochondrial function. As shown in Fig. 7, zVAD.fmk did not abrogate  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated cytochrome c release or ROS production under the same conditions where PS

exposure, PARP cleavage, and DNA fragmentation were blocked. In contrast, the caspase inhibitor effectively blocked cytochrome *c* release and ROS generation triggered by TNF $\alpha$  (Fig. 7, A and C).

To further probe mitochondrial function, the membrane potential-sensitive probe TMRE was used to detect mitochondrial membrane potential by flow cytometry. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment significantly enhanced the percentage of cells with reduced mitochondrial membrane potential, and zVAD.fmk did not block the decrease in mitochondrial membrane potential induced by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. TNF $\alpha$  treatment also enhanced the

percentage of cells with decreased mitochondrial membrane potential; however, in contrast to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, the effect of TNF $\alpha$  was completely blocked by zVAD.fmk (Fig. 7B).

Subcellular localization of cytochrome *c* protein was examined by fluorescence microscopy to confirm the finding that cytochrome *c* release can proceed independently of caspase activation after 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> treatment. In Fig. 8, cytochrome *c* fluorescence (*middle panels*) is presented alongside phase contrast (*top panels*) and Hoechst nuclear staining (*bottom panels*) to compare cytochrome *c* localization in individual viable and apoptotic cells. In vehicle-treated control cells, apoptotic morphology was not present, and cytochrome *c* staining was restricted to punctate cytoplasmic regions, consistent with mitochondrial localization (Fig. 8). After treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or TNF $\alpha$ , apoptotic cells identified by phase contrast and Hoechst nuclear staining exhibited chromatin condensation, nuclear fragmentation, and cytosolic vacuolization. In these apoptotic cells, diffuse cytoplasmic cytochrome *c* staining was detected throughout the cell, which obscured the nuclei, consistent with redistribution of cytochrome *c* from mitochondria to cytoplasm (21). Consistent with the immunoblotting data (Fig. 7A), treatment with zVAD.fmk failed to prevent 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated cytochrome *c* release, as demonstrated by persistence of diffuse cytoplasmic cytochrome *c* staining in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-plus zVAD.fmk-treated cells. However, zVAD.fmk did prevent the morphological signs of apoptosis, including chromatin condensation and nuclear fragmentation, consistent with its ability to block PS redistribution, PARP cleavage, and DNA fragmentation induced by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>.

Since zVAD.fmk did not block cytochrome *c* release or mitochondrial dysfunction induced by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, but did protect MCF-7 cells from morphological signs of apoptosis, including DNA fragmentation, we examined whether zVAD.fmk-treated cells actually remained viable and/or maintained clonogenic potential. As shown in Fig. 9, both zVAD.fmk and zDEVD.fmk caspase inhibitors rescued MCF-7 cells from TNF $\alpha$ -mediated cell death, as demonstrated by total cell num-

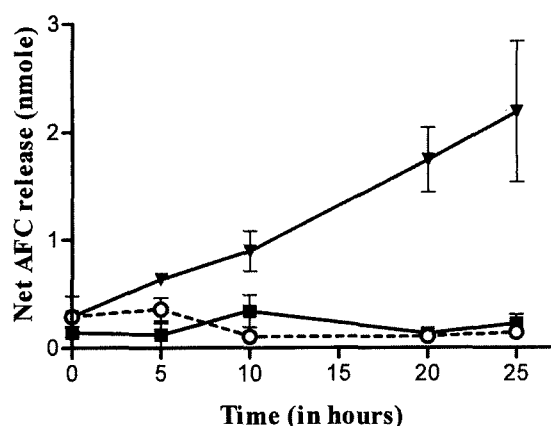


FIG. 6. Time course of DEVDase cleavage activity after treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or TNF $\alpha$  in MCF-7 cells. Cells were plated at a density of  $2 \times 10^5$  cells/150-mm dish. Two days after plating, the cells were pretreated with ethanol, 100 nM 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, or media only. Two days later, medium was replaced with ethanol (○), 100 nM 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (■), or 10 ng/ml TNF $\alpha$  (▼). Cytosolic extracts of cells harvested at the indicated time points were incubated with DEVD-AFC for 1 h at 37 °C and analyzed on a fluorescence spectrophotometer. Data represent mean  $\pm$  S.E. of two independent experiments performed in duplicate.

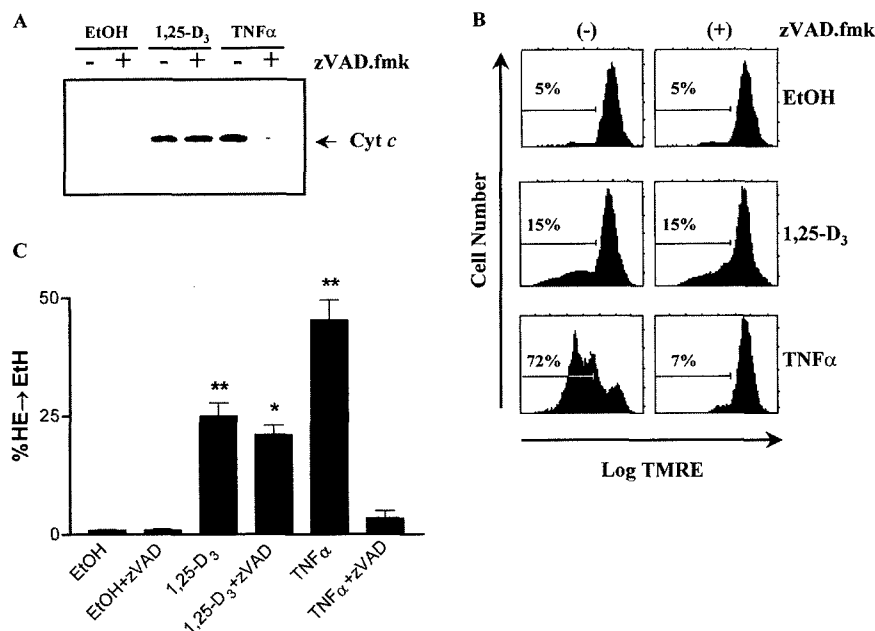


FIG. 7. Effects of caspase inhibitor on cytochrome *c* release, mitochondrial membrane potential, and ROS production after treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or TNF $\alpha$  in MCF-7 cells. A, cytochrome *c* release. S100 extracts prepared from cells treated as described in Fig. 4 were separated on SDS-PAGE and immunoblotted with cytochrome *c* (7H8.2C12) mouse monoclonal antibody. B, mitochondrial membrane potential. Cells plated and treated as described above were incubated with 1  $\mu$ M TMRE as described under "Experimental Procedures" and analyzed by flow cytometry. Data are expressed as the percentage of cells with reduced mitochondrial membrane potential. C, ROS production. Cells treated as described above were incubated with 4  $\mu$ M HE as described under "Experimental Procedures" and analyzed by flow cytometry. The data represent the percentage of cells positive for ROS. Each bar represents the mean  $\pm$  S.E. of two to four independent experiments. \*\*,  $p < 0.001$ ; \*,  $p < 0.01$ ; treated *versus* ethanol control as evaluated by analysis of variance. The results (A and B) are representative of at least three independent experiments.

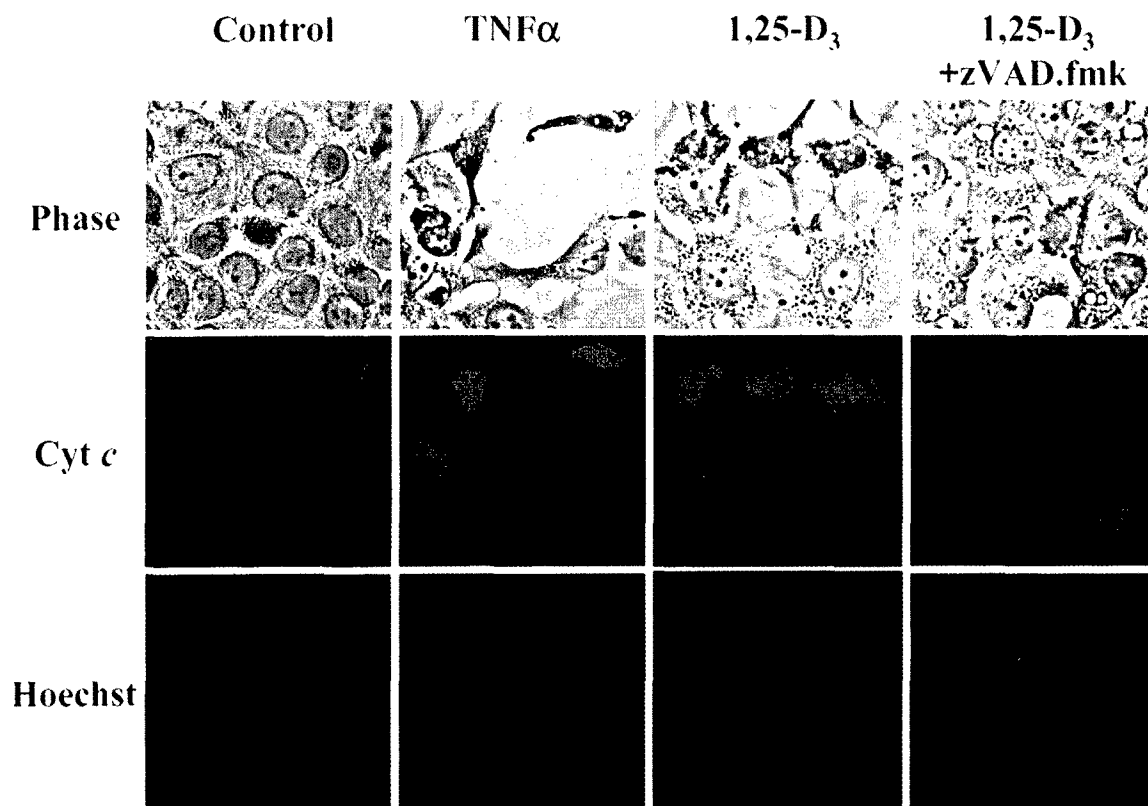


FIG. 8. Effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  on morphology and cytochrome *c* release in MCF-7 cells. Cells grown on Lab-Tek II chamber slides were treated with ethanol,  $100 \text{ nM } 1\alpha,25(\text{OH})_2\text{D}_3 \pm 25 \mu\text{M zVAD.fmk}$ , or  $2.5 \text{ ng/ml TNF}\alpha$ , fixed after 96 h (ethanol,  $1\alpha,25(\text{OH})_2\text{D}_3$ ) or 48 h ( $\text{TNF}\alpha$ ), immunostained with cytochrome *c* (6H2.B4) mouse monoclonal antibody, and visualized with Alexa-488-conjugated secondary antibody. Nuclei were counterstained with Hoechst 33258. The images were taken with an Olympus AX70 fluorescence microscope (original magnification,  $\times 400$ ). Top, phase contrast; middle, cytochrome *c* (green); bottom, Hoechst (blue). The results are representative of at least two independent experiments.

bers, with zVAD.fmk offering the greater protection. However, neither zVAD.fmk nor zDEVD.fmk caspase inhibitors could protect MCF-7 cells from  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated apoptosis, since the reduction in total cell number was not abrogated by either inhibitor (Fig. 9). Finally, the clonogenic potential was determined after treatment of cells with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$  in the presence or absence of zVAD.fmk followed by re-plating at limiting dilutions in fresh medium. In vehicle control-treated cultures, at least 1 out of 5 cells had the ability to produce clones. In  $\text{TNF}\alpha$ -treated cultures, clonogenicity was less than 1 out of 500 cells ( $f < 0.002$ ) but in the presence of zVAD.fmk, the frequency of cells with clonogenic potential was significantly increased ( $f \geq 0.2$ ). In  $1\alpha,25(\text{OH})_2\text{D}_3$  treated cultures, clonogenicity was less than 1 out of 50 cells ( $f < 0.02$ ), and this was not enhanced in the presence of zVAD.fmk.

#### DISCUSSION

Here we report for the first time that  $1\alpha,25(\text{OH})_2\text{D}_3$  induces apoptosis in MCF-7 cells by disruption of mitochondrial function, which is accomplished by translocation of Bax to mitochondria, and increased permeability of the outer mitochondrial membrane. Of particular interest, neither Bax translocation nor the mitochondrial disruption is induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  in a variant line of MCF-7 cells selected for resistance to  $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated apoptosis (13). Collectively, these data implicate an essential role for mitochondrial signaling in the induction of apoptosis by  $1\alpha,25(\text{OH})_2\text{D}_3$  and identify the pro-apoptotic protein Bax as an important downstream target of the VDR in MCF-7 cells.

In addition to Bax translocation, we report that treatment of MCF-7 cells with  $1\alpha,25(\text{OH})_2\text{D}_3$  induces cytochrome *c* release and ROS generation, events that have been observed in cells induced to undergo apoptosis by overexpression of Bax (22, 23).

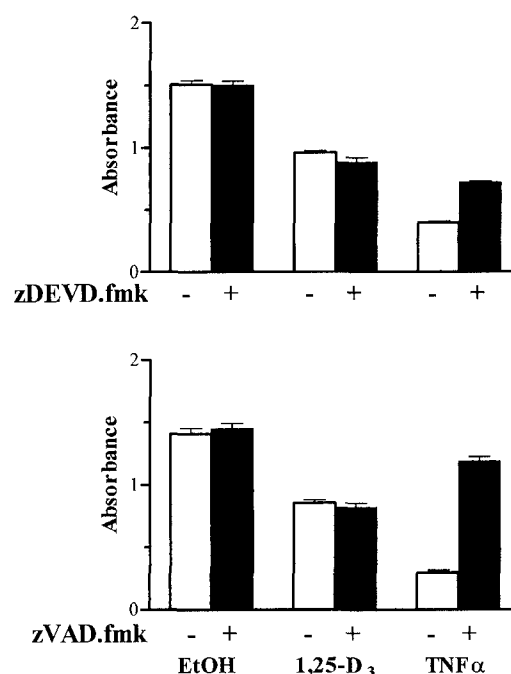


FIG. 9. Effect of caspase inhibitors on MCF-7 cell number after treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $\text{TNF}\alpha$ . Cells were seeded at a density of 1000 cells/well in 24-well plates. Two days after plating, cells were treated with ethanol,  $100 \text{ nM } 1\alpha,25(\text{OH})_2\text{D}_3$ , or  $10 \text{ ng/ml TNF}\alpha$  for 5 days  $\pm 25 \mu\text{M zVAD.fmk}$  (broad spectrum, bottom) or  $\text{zDEVD.fmk}$  (caspase-3/7-specific, top). Total cell number was determined by crystal violet assay as described under "Experimental Procedures." Data represent mean  $\pm$  S.E. of four replicate determinations. The results are representative of at least two independent experiments.



These data further support the concept that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis may be driven by Bax translocation. A role for the pro-apoptotic protein Bax in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis is consistent with previous studies that support a role for Bcl-2, the anti-apoptotic antagonistic partner of Bax, in mediating the effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on both breast and prostate cancer cells. Thus, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> down-regulates Bcl-2 (24, 25) and overexpression of Bcl-2 blocks 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-induced apoptosis (26, 27). Since Bcl-2 and Bax act antagonistically in the regulation of apoptosis, these data suggest that down-regulation of Bcl-2 in conjunction with translocation of Bax may be necessary for 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis. Further studies will be necessary to identify the signals generated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> that induce Bax translocation to mitochondria. Since events upstream of Bax translocation to mitochondria in response to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> are abrogated in the vitamin D<sub>3</sub>-resistant MCF-7 variant, comparison of early events in VDR signaling in these cells will be an important subject for future studies.

Examination of events downstream of mitochondria indicated that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> induced features of apoptosis associated with caspase activation, such as PARP cleavage, PS exposure, and DNA fragmentation. To determine whether caspase activation was required for 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis, we used the broad spectrum, cell-permeable caspase inhibitor zVAD.fmk. We observed that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> signaling on mitochondria does not require caspase activation, since zVAD.fmk was unable to block 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-induced cytochrome *c* release, decrease in mitochondrial membrane potential, or ROS production. Again, this is consistent with apoptosis driven by Bax translocation, which promotes cytochrome *c* release via caspase-independent pathways (28–31). Our data also complement that of Mathiasen *et al.* (27), who reported that inhibition of caspase activity by overexpression of CrmA, a cowpox-derived caspase inhibitor, or caspase inhibitory peptides (Ac-DEVD-CHO, Ac-IETD-CHO, and zVAD.fmk) did not block vitamin D<sub>3</sub>-mediated growth arrest or apoptosis.

Although caspase inhibition did not block mitochondrial events induced by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, zVAD.fmk did block events downstream of mitochondria such as PARP cleavage, external display of PS, and DNA fragmentation. These findings are similar to reports of Bax-induced apoptosis, where caspase inhibitors had no effect on Bax-induced cytochrome *c* release or mitochondrial disruption, but prevented cleavage of nuclear and cytosolic substrates and DNA degradation (28–31). However, our data conflict with that of Mathiasen *et al.* (27), who observed that zVAD.fmk did not block 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated DNA fragmentation in MCF-7 cells. This discrepancy may reflect differences in doses (1  $\mu$ M versus 25  $\mu$ M) or experimental design between the two studies. The lower dose of zVAD.fmk (1  $\mu$ M) used by Mathiasen *et al.* may have been insufficient to block mitochondrial-initiated caspases (caspase-9) (32).

The data presented in this paper indicate that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> triggers both caspase-independent and caspase-dependent pathways in MCF-7 cells, and suggest that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> can activate downstream effector caspases. Since cytochrome *c* release has been associated with autoactivation of procaspase-9, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> may activate caspase-dependent pathways via cytochrome *c* release. However, no DEVDase activity was detected in 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-treated cytoplasmic extracts, suggesting that other, possibly unidentified, effector caspases may be activated by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, or that caspase-dependent events occur at later stages in the apoptotic program. Although blocking caspase activation prevented some of the morphological aspects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis, MCF-7 cells were not rescued from death by zVAD.fmk. This finding is consistent

with reports that many cell types eventually die by a slower, non-apoptotic cell death if caspases are inactivated (8). These data support the concept that mitochondrial damage represents a cell death commitment step in the course of apoptosis induced by many stimuli (33), including 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>.

In summary, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> mediates apoptosis of MCF-7 cells through mitochondrial signaling, which involves ROS generation, and is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis, and caspase activation is not required for induction of cell death by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. Our data suggest distinct differences in the mechanisms of apoptosis induced by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and TNF $\alpha$ , since inhibition of caspases was able to rescue MCF-7 cells from TNF $\alpha$ -mediated, but not 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated, cell death. Although caspase inhibition blocked biochemical changes associated with caspase activation downstream of mitochondrial perturbations and loss of cytochrome *c*, the commitment of MCF-7 cells to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-mediated apoptosis is clearly caspase-independent.

**Acknowledgment**—We thank Thomas Waterfall for technical assistance.

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## Functions of $1\alpha,25$ -dihydroxyvitamin $D_3$ in mammary gland: from normal development to breast cancer

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### Abstract

This review examines the role of  $1\alpha,25(\text{OH})_2D_3$  (1,25D) and the vitamin  $D_3$  receptor in growth regulation of normal and transformed mammary epithelial cells. 1,25D exerts both anti-proliferative and pro-apoptotic functions in transformed mammary cells such as MCF-7. The anti-proliferative effects of 1,25D have been linked to suppression of growth stimulatory signals and potentiation of growth inhibitory signals, which lead to changes in cell cycle regulators such as p21, p27, cyclins and Rb. The pro-apoptotic effects of 1,25D involve alterations in the relative ratios of the bcl-2 family members which regulate mitochondrial integrity. In MCF-7 human breast cancer cells, 1,25D mediated apoptosis is associated with translocation of the pro-apoptotic protein Bax to the mitochondria, generation of reactive oxygen species, dissipation of the mitochondrial membrane potential and release of cytochrome c. These mitochondrial events trigger apoptosis in a caspase-independent manner, since caspase inhibitors do not rescue 1,25D treated cells from death. The potential role of 1,25D in growth and differentiation of normal mammary epithelial cells has been examined in VDR null mice. Initial data indicates a significant decrease in ductal differentiation in VDR null mice compared to age matched wild type mice, reflected as an increased number of undifferentiated terminal end buds in the VDR null mouse. These data suggest that 1,25D promotes differentiation during early mammary gland development. In summary, our studies suggest an expanding role for the vitamin  $D_3$  endocrine system in control of proliferation, differentiation and apoptosis of mammary epithelial cells. © 2001 Elsevier Science Inc. All rights reserved.

**Keywords:** Vitamin D; Estrogen; Mammary gland; Breast cancer; Apoptosis

### 1. Introduction

$1\alpha,25$ -Dihydroxyvitamin  $D_3$  (1,25D) is the biologically active form of vitamin  $D_3$ , a fat soluble vitamin identified as an anti-rachitic factor in the 1920s. In addition to its calcium regulatory properties, 1,25D is a negative growth regulator of breast cancer cells *in vivo* and *in vitro*. The ability of 1,25D to inhibit growth has consistently been observed in a variety of breast cancer cell lines, including those derived from human tumors [1,2]. Our lab initially demonstrated that 1,25D induces morphologic, biochemical and molecular features of apoptosis in MCF-7 human breast cancer cells [3]. Studies have shown that the nuclear vitamin  $D_3$  receptor (VDR), a ligand dependent transcription factor, is necessary (but not sufficient) for the induction of growth arrest and

apoptosis by 1,25D [4]. These observations predict that disturbances in VDR signaling could result in dysregulation of the mitotic and apoptotic pathways in normal mammary gland or in breast cancer. Disruption of VDR regulated pathways in normal mammary gland may predispose to transformation whereas abrogation of apoptosis in breast cancer cells may result in resistance to chemotherapeutic agents. Consistent with this possibility, several studies have reported associations between circulating vitamin  $D_3$  metabolites or VDR polymorphisms and breast cancer risk or disease activity [5–9]. In this brief review, we will focus on three specific areas of investigation related to vitamin  $D_3$  signaling in relation to proliferation and apoptosis of mammary epithelial cells:

*What mechanisms are involved in vitamin  $D_3$  mediated growth regulation?*

*What factors regulate VDR expression in breast cancer cells?*

*Does vitamin  $D_3$  play a role in normal mammary gland function?*

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## 2. Growth regulation of breast cancer cells by $1\alpha,25$ -dihydroxyvitamin $D_3$

### 2.1. Vitamin $D_3$ compounds are potent growth regulators of breast cancer cells

Numerous studies have established that  $1,25D$  modulates cell cycle, differentiation, invasion and apoptosis of breast cancer cells in vitro (reviewed in [10]). These and other data have generated considerable interest in development of structural analogs of vitamin  $D_3$  with minimal calcemic side effects as potential therapeutic agents for human cancers [11]. A phase I trial of EB1089, an analog developed by LEO Pharmaceuticals, Ballerup, Denmark, supported the concept that vitamin  $D_3$  analogs display less calcemic properties than  $1,25D$  in patients with advanced breast cancer [12]. Several low calcemic vitamin  $D_3$  analogs have indeed shown efficacy in prevention and/or treatment of a variety of tumor types, including breast. For example, EB1089 inhibited growth of NMU-induced rat mammary tumors [13] and induced regression of human breast cancer xenografts in nude mice [14,15]. Vitamin  $D_3$  analogs have also been shown to enhance the sensitivity of breast cancer cells and/or tumors to a variety of therapeutic regimens, including anti-estrogens, retinoids, radiation and chemotherapeutic drugs [15–19]. Rational design of more effective vitamin  $D_3$  analogs to be used in primary or adjunctive treatment regimens for breast cancer relies heavily on an understanding of the mechanisms by which  $1,25D$  and the VDR regulate the growth of transformed cells.

### 2.2. Mechanisms of cell cycle regulation by $1,25D$

Treatment of estrogen dependent MCF-7 human breast cancer cells with  $1,25D$  induces cell cycle arrest in  $G_0/G_1$  which has been associated with up-regulation of the cell cycle inhibitors p21 and p27, down regulation of cyclins A and D1, decreased cdk2 activity and de-phosphorylation of the retinoblastoma protein [20–24]. The effects of  $1,25D$  on cell cycle have been linked to down-regulation of growth promoting signals, such as IGF-1, as well as up-regulation of negative growth regulators, such as  $TGF\beta$  [24–29]. While the majority of data have been generated with the well-characterized, estrogen receptor positive MCF-7 cells,  $1,25D$  also exerts anti-proliferative effects in estrogen independent cell lines [1,16,30]. As discussed further in Section 3 below, however, sensitivity to  $1,25D$  and VDR expression tend to be higher in breast cancer cells which express the estrogen receptor than in those that do not [31].

### 2.3. $1\alpha,25$ -Dihydroxyvitamin $D_3$ induced apoptosis in breast cancer cells

In addition to its anti-proliferative effects,  $1,25D$  induces morphologic and biochemical features of apoptosis in breast cancer cells [3,23,32–35]. MCF-7 cells treated with 100 nM

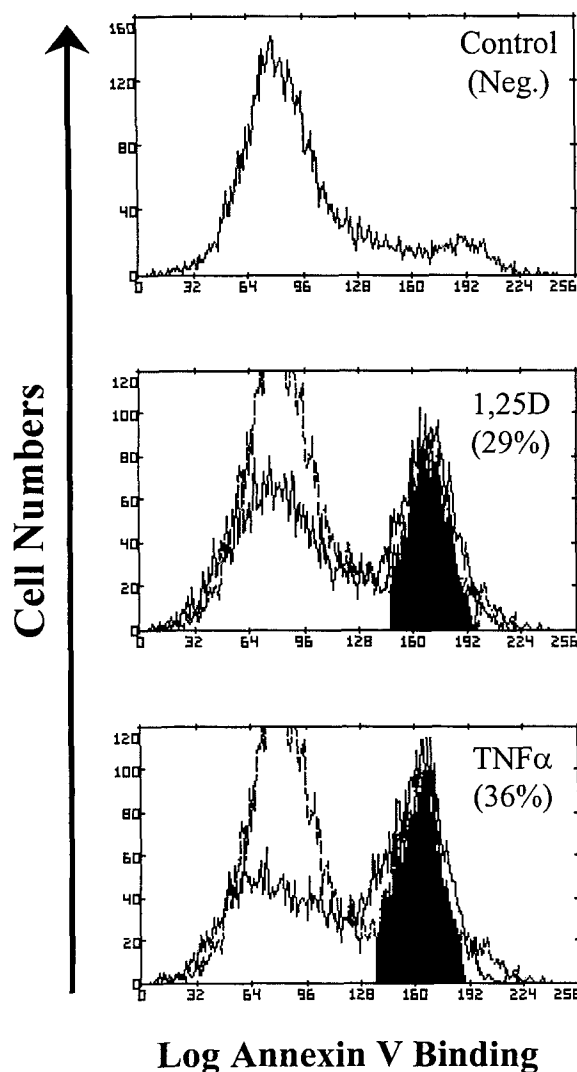


Fig. 1. Exposure of phosphatidylserine (PS) in MCF-7 cells after treatment with  $1,25D$  or  $TNF\alpha$ . PS exposure was analyzed by flow cytometry of FITC-conjugated annexin V in MCF-7 cells treated with 100 nM  $1,25D$ , 1 ng/ml  $TNF\alpha$  or ethanol vehicle. Numbers in upper right corner of each panel indicate the percentage of positive cells after negative subtraction of data generated with vehicle treated cells.

$1,25D$  exhibit cell shrinkage, chromatin condensation and DNA fragmentation, indicative of apoptosis [3]. Re-orientation of phosphatidylserine (PS) to the exterior of the cell, another marker of apoptosis, occurs in  $1,25D$  treated MCF-7 cells and can easily be measured by flow cytometry of annexin V binding (Fig. 1). Our lab has also reported that treatment of nude mice bearing MCF-7 xenografts with the vitamin  $D_3$  analog EB1089 markedly inhibits tumor growth via induction of apoptosis in tumor epithelial cells [13]. Up-regulation of apoptotic related proteins, such as clusterin, cathepsin B and  $TGF\beta$ , has been reported in MCF-7 cells undergoing  $1,25D$  mediated apoptosis [3,22,34]. Furthermore,  $1,25D$  treatment has been shown to enhance cellular sensitivity to other triggers of apoptosis, such as anti-estrogens,  $TNF\alpha$ , radiation and chemotherapeutic agents

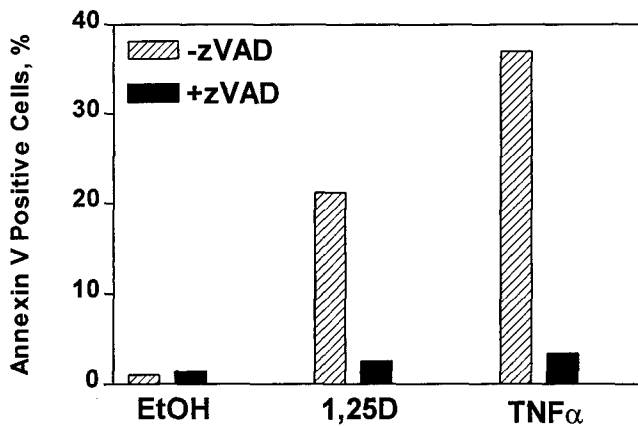


Fig. 2. Effect of caspase inhibitors on PS exposure in 1,25D and TNF $\alpha$  treated MCF-7 cells. MCF-7 cells were treated with 100 nM 1,25D or 1 ng/ml TNF $\alpha$  in the presence or absence of the cell permeable caspase inhibitor zVAD-fmk (25  $\mu$ M) and analyzed for annexin V binding by flow cytometry. Data are expressed as the percentage of annexin V positive cells after negative subtraction of data generated with vehicle treated cells.

[15,17,19,35]. It is not quite clear whether these synergistic effects result from interactions of 1,25D with agonist-specific signaling pathways or whether 1,25D impacts on components of a common apoptotic pathway.

In order to examine the mechanism of 1,25D mediated apoptosis in breast cancer cells, we have compared specific intracellular events in MCF-7 cells after treatment with 1,25D or TNF $\alpha$ . TNF $\alpha$  was chosen as a positive control since this cytokine is known to induce apoptosis in MCF-7 cells via a well defined pathway triggered by TNFR1, a cell surface receptor. TNFR1 is a member of the 'death domain' containing family of apoptotic receptors whose signaling is linked to activation of caspases. Caspases represent a family of pre-existing cysteine proteases which become activated during apoptosis. Cleavage of specific substrates by caspases during apoptosis promotes the degradation of key structural proteins, leading to cellular condensation and DNA fragmentation.

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In association with morphologic evidence of apoptosis and PS exposure, we have observed that MCF-7 cells treated with 1,25D exhibit cleavage of PARP, a known nuclear substrate of caspase-3, suggesting that vitamin D<sub>3</sub> mediated apoptosis may involve activation of caspases. Since studies have indicated that caspase activation is not required for all forms of apoptosis, we have examined whether 1,25D mediated apoptosis in MCF-7 cells is caspase dependent. As shown in Fig. 2, cell permeable caspase inhibitors such as zVAD are effective in blocking the re-orientation of PS (again measured by annexin V binding) in response to both 1,25D and TNF $\alpha$ . We have also observed that blocking caspase activation prevents 1,25D induced PARP cleavage and DNA fragmentation in MCF-7

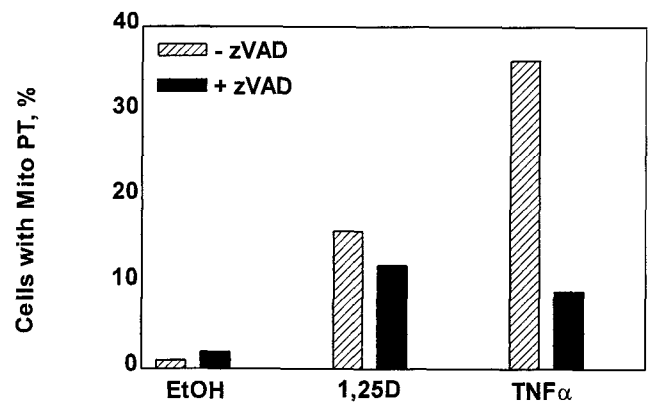


Fig. 3. Effect of 1,25D and TNF $\alpha$  on mitochondrial membrane permeability in MCF-7 cells. MCF-7 cells were treated with ethanol vehicle, 100 nM 1,25D or 1 ng/ml TNF $\alpha$ , in the presence or absence of the cell permeable caspase inhibitor zVAD-fmk (25  $\mu$ M), and mitochondrial membrane permeability was measured by flow cytometry after incubation with the fluorescent dye, DiOC<sub>6</sub>. Data are expressed as the percentage of cells displaying reduced fluorescence due to dissipation of the mitochondrial membrane potential after negative subtraction of data generated with vehicle treated cells.

cells (not shown). However, assessment of viable cell numbers indicates that while caspase inhibitors can block late events in the apoptotic process (such as PS exposure, PARP cleavage and DNA fragmentation), caspase inhibition does not actually rescue cells from 1,25D mediated cell death [36]. Likewise, over-expression of CrmA, a viral inhibitor of caspases, does not prevent 1,25D induced apoptosis in MCF-7 cells [36]. These data suggest that although caspases may be activated during 1,25D mediated apoptosis, as evidenced by the ability of caspase inhibitors to block the late stages of apoptosis, the commitment of 1,25D treated MCF-7 cells to death can occur even if caspase activation is prevented.

A newly recognized intracellular feature of apoptosis is disruption of the mitochondrial inner membrane potential, a phenomenon termed permeability transition (PT). During apoptosis, PT is associated with release of several factors, including cytochrome c, which activate downstream events culminating in DNA fragmentation and apoptotic morphology [37]. We have observed that apoptosis in MCF-7 cells treated with 1,25D is associated with dissipation of mitochondrial membrane potential, as measured by flow cytometry using a fluorescent dye, DiOC<sub>6</sub> (Fig. 3). Pre-treatment with the caspase inhibitor zVAD does not block the effects of 1,25D on mitochondrial PT. In contrast, the effect of TNF $\alpha$  on mitochondrial PT is markedly blunted by zVAD pretreatment in MCF-7 cells (Fig. 3). These data indicate that while similarities exist between 1,25D and TNF $\alpha$  induced apoptosis with respect to mitochondrial events, apoptosis mediated by these two triggers are differentially sensitive to caspase inhibitors.

Since one consequence of the mitochondrial PT is the release of cytochrome c, we have examined expression of cytochrome c in cytosol by Western blotting in MCF-7 cells

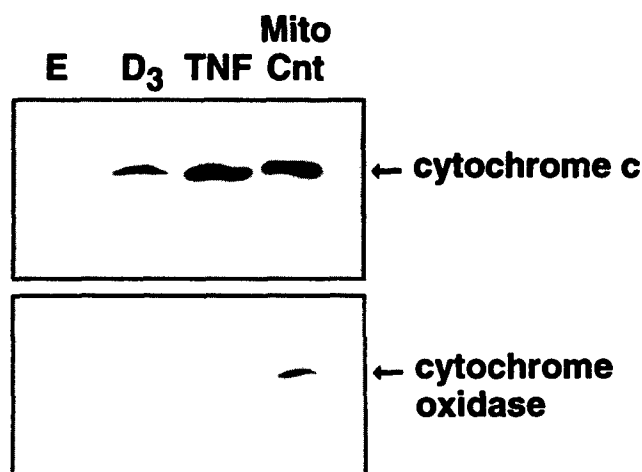


Fig. 4. Cytosolic cytochrome *c* in MCF-7 cells after treatment with 1,25D and TNF $\alpha$ . Expression of cytochrome *c* and cytochrome oxidase was assessed by Western blotting of cytosolic fractions derived from MCF-7 cells treated with ethanol vehicle (E), 1,25D (D<sub>3</sub>) or TNF $\alpha$ . Note that cytochrome *c* is not present in cytosolic fractions of vehicle treated cells, but is present in 1,25D or TNF $\alpha$  treated cells. Cytochrome oxidase, another mitochondrial protein, is not detected in cytosolic fractions from any cells. Both cytochrome oxidase and cytochrome *c* are detected in mitochondrial extract (Mito Cnt), which was included as a positive control.

treated with 1,25D or TNF $\alpha$ . As demonstrated in Fig. 4, cytochrome *c* is not detected in the cytosolic fraction of control (ethanol treated) cells, but is readily detected in the cytosolic fraction of either 1,25D or TNF $\alpha$  treated cells. Additional studies in our lab have indicated that the release of cytochrome *c* is detected within 48 h of 1,25D exposure and precedes the onset of apoptotic morphology in MCF-7 cells. Mitochondrial disruption during apoptosis of MCF-7 cells has also been associated with oxidative stress, and we have observed a time dependent increase in the generation of reactive oxygen species (ROS) in 1,25D treated MCF-7 cells (Fig. 5). Consistent with the lack of effect of caspase inhibitors on 1,25D induced mitochondrial PT, zVAD does not prevent either the release of cytochrome *c* or ROS generation in response to 1,25D treatment in MCF-7 cells. Thus, mitochondrial disruption (membrane permeability dissipation, cytochrome *c* release and ROS generation) persists in 1,25D cells even in the presence of caspase inhibitors. In contrast, mitochondrial PT, cytochrome *c* release and ROS generation in response to TNF $\alpha$  are blocked by caspase inhibition. Thus, our results suggest a model in which apoptosis induction by 1,25D in MCF-7 cells is distinct from that induced by TNF $\alpha$ , as 1,25D triggers mitochondrial disruption, cytochrome *c* release and ROS generation via caspase independent pathways (Fig. 6).

The model outlined in Fig. 6 predicts that 1,25D mediated apoptosis would be modulated by the Bcl-2 family of pro- and anti-apoptotic proteins, which act at the mitochondrial level to regulate the release of cytochrome *c* [38]. Consistent with this prediction, we have found that 1,25D treatment induces redistribution of the pro-apoptotic Bcl-2 family member, Bax, from the cytosol to the mitochondria

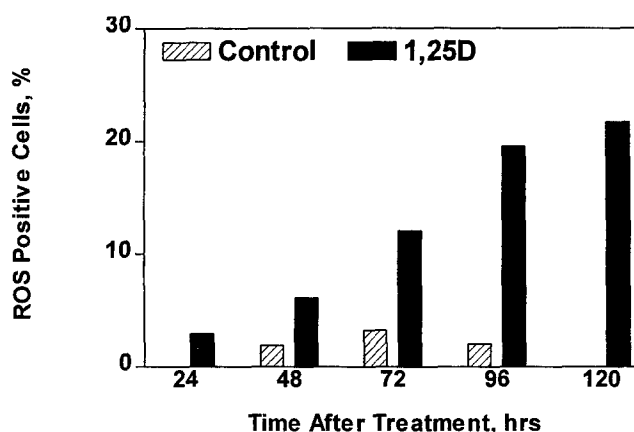


Fig. 5. Generation of reactive oxygen species (ROS) during apoptosis of 1,25D treated MCF-7 cells. Production of reactive oxygen species (ROS) was measured in MCF-7 cells treated with 100 nM 1,25D or ethanol vehicle for up to 120 h. ROS production was measured by flow cytometry of hydroethidine fluorescence. The percentage of cells positive for ROS was calculated by negative subtraction of data derived from vehicle treated cells at time zero.

in MCF-7 cells. In addition, 1,25D treatment of MCF-7 cells has been shown to down regulate the anti-apoptotic protein Bcl-2 [22,34] and overexpression of Bcl-2 renders MCF-7 cells resistant to 1,25D mediated apoptosis [36]. These data suggest that sensitivity to 1,25D mediated apoptosis is determined by the relative expression and/or sub-cellular localization of the Bcl-2 family of pro- and anti-apoptotic proteins.

In summary, 1,25D mediates apoptosis of MCF-7 cells through mitochondrial signaling which likely involves oxidative stress and is regulated by the Bcl-2 family of apoptotic regulators. This pathway may be amplified or facilitated by caspases, but caspase activation is not required for 1,25D mediated apoptosis. Additional studies will be necessary to determine whether modulation of these apoptotic signals by 1,25D are cell type specific, and whether structural analogs of 1,25D can be identified which preferentially trigger these events. Perhaps most important will be identification of the specific genes regulated by the VDR which impact on mitochondrial integrity.

### 3. Regulation of the VDR in breast cancer cells

VDR abundance is affected by many physiological factors and is achieved through a variety of mechanisms, including alterations in transcription and/or mRNA stability, post-translational effects and ligand induced stabilization (reviewed in [39]). Expression of the VDR in cultured cells and in vivo is regulated by many physiological agents, including 1,25D itself, estrogens, glucocorticoids and retinoids. Thus, cellular sensitivity to 1,25D mediated growth regulation may reflect the activity of other hormone signaling pathways through their impact on VDR expression.

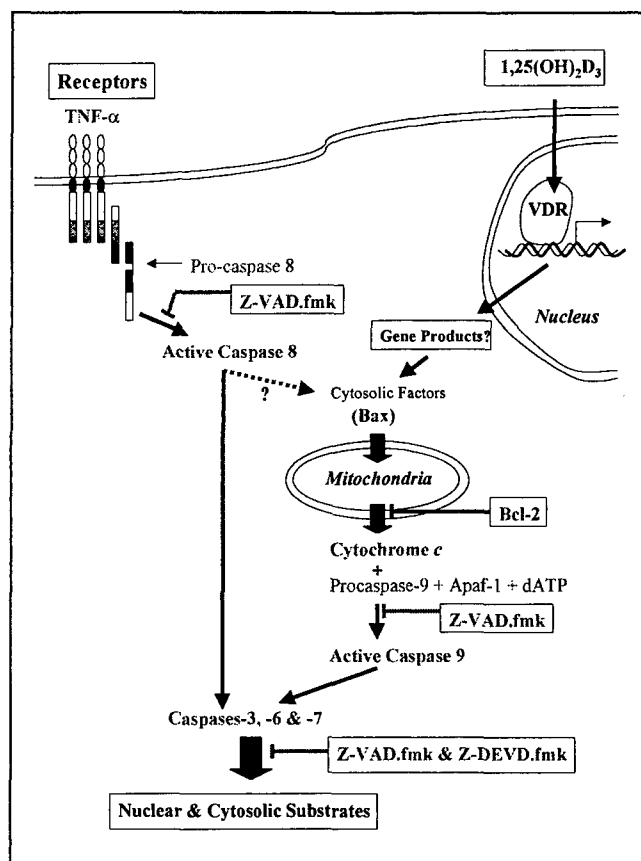


Fig. 6. Model for 1,25D induced apoptosis in MCF-7 cells. This model suggests that 1,25D acts through the nuclear VDR to modulate gene products which alter Bax subcellular distribution and mitochondrial events, culminating in the release of cytochrome c. These initial events induced by 1,25D are caspase independent and commit the cell to the death program. In contrast, TNF $\alpha$  acts through its cell surface receptor and mediates mitochondrial disruption via caspase activation. See text for additional details.

Unfortunately, little attention has been paid to identifying factors which enhance or depress sensitivity of breast cancer cells to 1,25D via manipulation of VDR levels. Our own studies have suggested that regulation of the VDR is distinctly different in MCF-7 cells selected for 1,25D resistance compared to parental MCF-7 cells (4 and unpublished data). In particular, very limited information exists on VDR regulation in vivo, and no studies have examined regulation of the VDR in animal models of breast cancer. An important precedent for enhanced tumor sensitivity to 1,25D therapy via cell type specific regulation of VDR by dexamethasone has been reported for a murine model of squamous carcinoma [40]. Although speculative at this point, it is possible that modulation of tumor VDR levels underlies the synergistic effects observed with combination therapies involving vitamin D<sub>3</sub> analogs for breast cancer.

In breast cancer cells, interactions between estrogen receptor and VDR in growth regulation have been proposed [10,15]. We have reported that breast cancer cells selected for anti-estrogen resistance and those negative for estrogen receptor retain sensitivity to 1,25D mediated cell cycle

arrest and apoptosis [10,15,41], although, in general, breast cancer cells that express functional estrogen receptor display enhanced sensitivity to 1,25D mediated growth inhibition [31]. Comparison of a panel of breast cancer cells indicated that estrogen receptor positive cells tended to express higher levels of VDR than estrogen receptor negative cells [31]. Furthermore, in vitro studies have indicated that estrogen up-regulates the VDR, and anti-estrogens such as tamoxifen down regulate the VDR, in estrogen receptor positive breast cancer cells [4,42]. Collectively, these data support the concept that estrogen and anti-estrogens are important regulators of VDR expression in breast cancer cells.

Regulation of the VDR by estrogens and anti-estrogens has considerable clinical implications arising from the potential use of selective estrogen response modifiers (SERMs) and vitamin D<sub>3</sub> analogs for prevention and/or treatment of breast cancer and osteoporosis, diseases most commonly present in post-menopausal women [11,43]. The efficacy and toxicity of vitamin D<sub>3</sub> analogs is determined, in part, by the level of VDR in target tissues, and thus it will be important to determine the degree to which estrogen status influences VDR abundance in different 1,25D target cells in vivo (i.e. breast, bone, uterus). In this respect, it will also be important to assess whether novel SERMs currently utilized for estrogen replacement therapy of post-menopausal women, such as raloxifene [43], act as estrogen agonists or antagonists in regulation of VDR expression. This is especially pertinent in view of the increasing interest in the use of SERMs for breast cancer prevention.

#### 4. Vitamin D<sub>3</sub> and the VDR in normal mammary gland

##### 4.1. Vitamin D<sub>3</sub> receptor in mammary gland

The VDR in mammary gland is dynamically regulated during pregnancy and lactation [44]; however, little is known about its specific functions or regulation in this tissue. The VDR is expressed at low levels in pubertal mammary gland explants and is upregulated in response to the differentiating inducing hormones cortisol, prolactin and insulin [45]. Mammary glands from vitamin D<sub>3</sub> deficient mice display impaired induction of  $\beta$ -casein and  $\alpha$ -lactalbumin in vitro compared to glands from vitamin D<sub>3</sub> replete mice [46]. Addition of 1,25D to mammary gland explants increased VDR expression [47] and enhanced calcium uptake [48]. These studies suggest that 1,25D may play a role in differentiation and milk production in the mammary gland.

Animal studies have demonstrated that dietary vitamin D<sub>3</sub> can negate the promoting effect of fat on mammary tumorigenesis [49,50], and that treatment with a vitamin D<sub>3</sub> analog can prevent the development of NMU induced mammary tumors [51]. In mammary gland explants, treatment

with 1,25D prevented development of pre-neoplastic lesions following DMBA treatment [47], indicating a direct inhibitory effect of 1,25D on mammary gland sensitivity to transformation. Collectively, these data indicate that vitamin D<sub>3</sub> and its analogs act to suppress tumorigenesis of normal mammary epithelial cells, however, the mechanisms involved in these protective effects have not been extensively investigated to date.

#### 4.2. Investigation of mammary gland development in VDR null mice

To begin to address the physiological role of vitamin D<sub>3</sub> signaling in normal mammary gland, we have examined mammary gland development in VDR null ('knockout') mice [52]. The VDR null mice are phenotypically normal at birth [52,53], but the switch from a calcium rich milk-based diet to a chow diet at weaning precipitates progressive hypocalcemia, bone abnormalities and growth retardation. Normalization of mineral ion homeostasis by feeding a diet high in calcium, phosphorus and lactose prevents the development of hyperparathyroidism, rickets and growth retardation, suggesting that these effects of VDR ablation are secondary to impaired calcium metabolism rather than receptor deficiency per se [52]. Murine mammary gland develops after weaning, and involves extensive rounds of proliferation and apoptosis in specialized structures called terminal end buds. Since the VDR is expressed in neonatal murine mammary gland [47], we hypothesize that dysregulation of VDR target genes in mammary gland could alter proliferation and apoptosis sufficiently to alter mammary gland development and possibly even predispose mammary epithelial cells to transformation.

Whole mount analysis was used to assess post-natal ductal development and branching in VDR  $+/+$  and  $-/-$  mice fed the high calcium 'rescue' diet to normalize calcium homeostasis [52]. Comparison of mammary whole mounts from 4 weeks old  $+/+$  and  $-/-$  VDR mice revealed little differences in ductal development due to genotype, indicating that the initial round of branching morphogenesis proceeds normally in VDR null mice. However, comparison of mammary whole mounts from 6 weeks old  $+/+$  and  $-/-$  VDR mice (Fig. 7) revealed differences in the extent of ductal differentiation as well as the degree of branching. In particular, the number of undifferentiated terminal end bud structures is higher in glands from  $-/-$  VDR mice compared to  $+/+$  mice. Persistence of terminal end buds in glands from  $-/-$  mice may have functional significance since these undifferentiated structures are the targets for chemical carcinogens such as DMBA. Quantitation of proliferation and apoptosis in terminal end buds from normal and VDR null mice will indicate whether 1,25D impacts on these growth regulatory pathways in vivo, and if so, whether dysregulation of these pathways due to lack of the VDR alters mammary gland sensitivity to transformation.

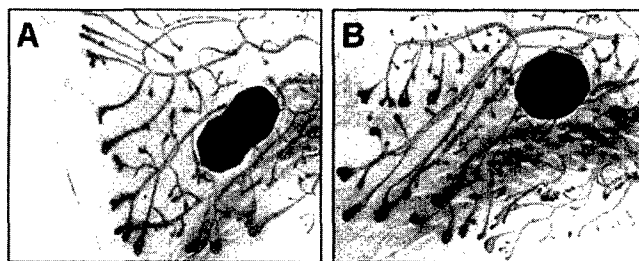


Fig. 7. Mammary gland development in wild type and VDR null mice. Inguinal mammary glands were removed from six week old wild type ( $+/+$ ) and VDR null ( $-/-$ ) mice fed a high calcium 'rescue' diet, fixed and stained with carmine alum. Whole mounts of the entire mammary fat pad were viewed and photographed to visualize ductal development and branching. The dark rounded structure in the center is the inguinal lymph node, which becomes surrounded by mammary ducts as the gland matures.

## 5. Summary

This review supports an expanding role for the vitamin D<sub>3</sub> endocrine system in control of proliferation, differentiation and apoptosis of mammary epithelial cells. Modulation of these critical cell regulatory pathways by 1,25D appears to suppress tumorigenesis in normal mammary cells, and can be exploited to inhibit growth of established breast cancers. While numerous VDR target genes have been linked to these growth regulatory pathways in breast cancer cells, it is not clear if the same genes are regulated by 1,25D in normal mammary cells. New in vivo model systems, in particular, the VDR null mice, combined with powerful genomic approaches, to search for VDR regulated genes in mammary gland, can be exploited to address this question. More information of the molecular regulation of the VDR is needed in order to design strategies for enhancing tumor sensitivity to vitamin D<sub>3</sub> analogs via manipulation of the VDR. In this respect, the potential regulation of the VDR by SERMs, such as tamoxifen and raloxifene, which have been shown to prevent breast cancer, needs to be clarified. Vitamin D<sub>3</sub> analogs which trigger specific subsets of vitamin D<sub>3</sub> responses, or exert cell specific effects, would have tremendous clinical value not only in cancer prevention and/or treatment, but also in management of several other significant human diseases.

## Acknowledgments

Research in the author's laboratory is funded by the National Cancer Institute (CA69700), the American Institute for Cancer Research (98A100) and the USDA CREES program (99–35200–7614). C.J. Narvaez is supported by a Post Doctoral Fellowship from the Department of Defense (17–97–1–7183). Glen Zinser is supported by a BBMB Graduate Student Fellowship from the University of Notre Dame.

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## ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS

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**Introduction** 1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, acts through the nuclear vitamin D receptor (VDR) and is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo*. Our lab has shown that 1,25-(OH)<sub>2</sub>D<sub>3</sub> induces morphological and biochemical markers of apoptosis (chromatin and nuclear matrix condensation, and DNA fragmentation) in MCF-7 breast cancer cells (1). The precise mechanism of how 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its nuclear receptor, the VDR, mediate apoptosis is poorly understood.

We examined the 1,25-(OH)<sub>2</sub>D<sub>3</sub> signaling pathway downstream of the VDR in order to identify specific intracellular events involved in 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis and to characterize events which are blocked in MCF-7<sup>D3Res</sup> cells (a vitamin D<sub>3</sub>-resistant variant)(2). In particular, the effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis on mitochondrial function and caspase activity were studied and compared to the effects of TNF $\alpha$ . TNF $\alpha$  was chosen as a positive control since this cytokine induces apoptosis in MCF-7 cells by means of a well-defined pathway triggered by TNFR1, a cell surface death receptor whose signaling results in caspase activation and disruption of mitochondrial function (3). Caspases are a family of evolutionarily conserved cysteine proteases that become activated upon proteolytic cleavage, and are responsible for cell disassembly. Mitochondria play a central role in controlling cell death. Translocation of Bax from cytosol to mitochondria, release of cytochrome c, and activation of caspases may initiate disruption of mitochondrial function (4,5). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspases and nuclease activators released from mitochondria leading to the ultimate destruction of the cell.

While the role of caspases in apoptosis triggered by cell surface death receptors such as TNFR1 has been well established, it is not clear if apoptosis triggered by nuclear receptors such as the VDR is mediated via similar caspase dependent pathways. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to determine the involvement of caspase-dependent proteolysis in 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis.

**Results and Discussion** Disruption of mitochondrial function is one of the primary events that occur during apoptosis. Translocation of Bax to the mitochondrial outer membrane has been implicated in induction of apoptosis.

and  $\text{TNF}\alpha$  in MCF-7 cells (Figure 1). Not only was Bax translocated to mitochondria, but it was also cleaved from 21 kDa to 18 kDa. This observation is consistent with other reports of Bax cleavage during drug-induced apoptosis (6). Bax translocation to mitochondria and apoptosis in response to  $\text{TNF}\alpha$  can be triggered in MCF-7<sup>D3Res</sup> cells indicating that Bax functions appropriately during apoptosis induced by agents other than  $1,25\text{-(OH)}_2\text{D}_3$  (Figure 1).

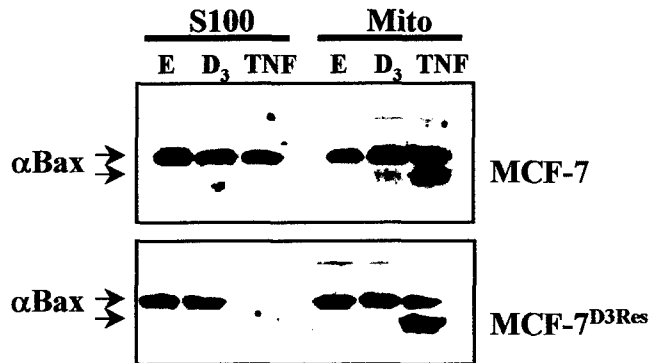


Figure 1 Subcellular distribution of Bax in MCF-7 and MCF-7<sup>D3Res</sup> cells after treatment with  $1,25\text{-(OH)}_2\text{D}_3$  or  $\text{TNF}\alpha$ .

Translocation of Bax to mitochondria is associated with subsequent release of cytochrome c, events that are considered to be commitment points for activating apoptosis. Cytochrome c normally resides within the intermembrane space of live cells. Cytochrome c is not detected in the cytosolic fraction of vehicle treated control cells. However,  $1,25\text{-(OH)}_2\text{D}_3$  induces redistribution of cytochrome c from mitochondria to cytosol as early as 48 hrs in MCF-7 cells,

before any morphological apoptotic events are detected. By contrast,  $\text{TNF}\alpha$ , but not  $1,25\text{-(OH)}_2\text{D}_3$ , induces release of cytochrome c in MCF-7<sup>D3Res</sup> cells.

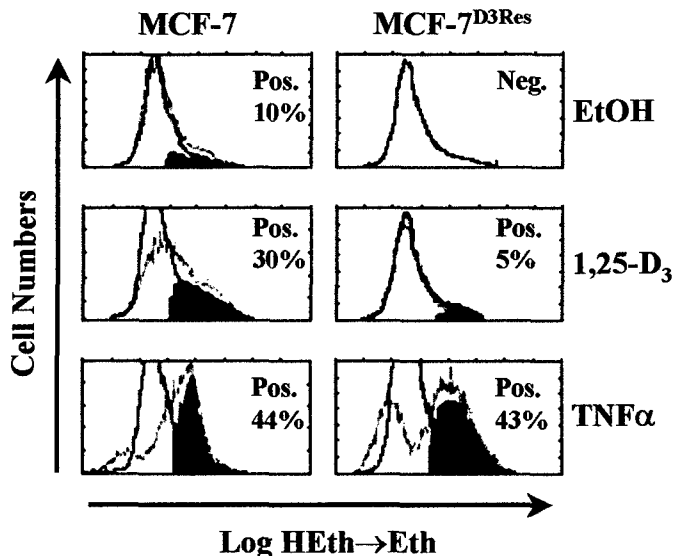


Figure 2 ROS production in MCF-7 or MCF-7<sup>D3Res</sup> cells after treatment with  $1,25\text{-(OH)}_2\text{D}_3$  or  $\text{TNF}\alpha$ .

Long term exclusion of cytochrome c from the electron transport chain can lead to impairment of proton flow, and generation of reactive oxygen species (ROS) due to incomplete reduction of molecular oxygen (7). Hence, mitochondrial generation of ROS in response to apoptotic stimuli was examined. By using flow cytometric techniques, production of superoxide anion was assessed by the degree of oxidation of hydroethidine to ethidium, a DNA stain that fluoresces red upon DNA intercalation. MCF-7 cells,

fluoresces red upon DNA intercalation. MCF-7 cells, but not MCF-7<sup>D3Res</sup>, produced ROS in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, whereas TNF $\alpha$  induced ROS production in both cell lines (Figure 2).

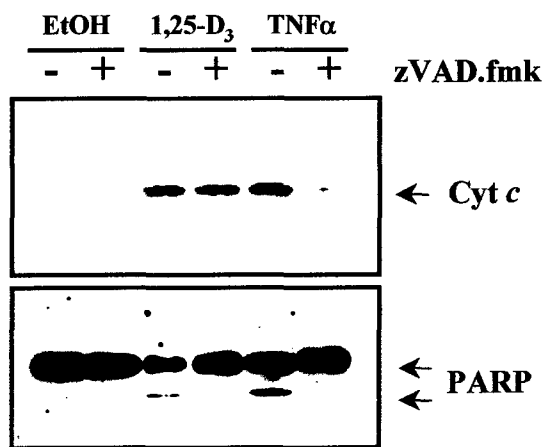


Figure 3 Expression of cytochrome c and PARP in MCF-7 cells (in the presence or absence of zVAD.fmk).

Cytochrome c release into the cytosol triggers caspase activity downstream of mitochondria. In order to determine the involvement of caspase-dependent proteolysis in 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis, we used a broad-spectrum cell permeable inhibitor (zVAD.fmk) in MCF-7 cells. Proteolytic activity associated with caspases was analyzed by three different methods. First, caspase activity was detected by immunoblot analysis of the cleavage of an endogenous caspase substrate, poly (ADP-ribose) polymerase (PARP). PARP was

cleaved in the presence of both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and TNF $\alpha$ . The cleavage was blocked by zVAD.fmk (Figure 3). The second and third methods utilized flow cytometry to analyze phosphatidylserine (PS) exposure and DNA fragmentation, respectively, which others have shown can be provoked by caspases (8). Annexin V-FITC is a convenient probe for monitoring changes in the distribution of PS in the plasma membrane during apoptosis. Both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and TNF $\alpha$  induced PS exposure in MCF-7 cells, which was blocked by zVAD.fmk treatment. DNA fragmentation was assessed by incorporation of bromodeoxyuridine by terminal transferase and detection by anti-bromodeoxyuridine antibody conjugated to FITC. Both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and TNF $\alpha$  induced DNA fragmentation, which was completely blocked by zVAD.fmk. However, TNF $\alpha$ , but not 1,25-(OH)<sub>2</sub>D<sub>3</sub>, induced DEVDase (caspase-3/7) cleavage activity in MCF-7 cells. This observation indicates that other, or as yet unidentified, effector caspases may be responsible for 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated PARP cleavage, PS exposure, or DNA fragmentation.

Since zVAD.fmk blocked caspase activity downstream of mitochondria, we examined the effects of the caspase inhibitor on cytochrome c release and mitochondrial activity. The caspase inhibitor had no effect on 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced cytochrome c release (Figure 3), decrease in mitochondrial membrane potential, or ROS production. However, caspase inhibitor was able to block all mitochondrial events in response to TNF $\alpha$ . This demonstrates that TNF $\alpha$  induced cell death occurs by a caspase-dependent mechanism consistent with a role for caspase-8 in triggering cytochrome c release.

Since zVAD.fmk was unable to block cytochrome *c* release and mitochondrial dysfunction in response to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, we determined the effect of caspase inhibitor on cell death and clonogenic potential. Both zVAD.fmk and zDEVD.fmk caspase inhibitors protected MCF-7 from TNF $\alpha$  cell death with zVAD.fmk exhibiting the greatest response. However, neither caspase inhibitor could protect MCF-7 cells from 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis since the reduction in cell number was not blocked by these inhibitors. In addition, MCF-7 cells treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> lost their clonogenic potential even when they were treated in the presence of zVAD.fmk. This suggests that the activation of caspases by 1,25-(OH)<sub>2</sub>D<sub>3</sub> occurs subsequent to the events that commits the cells to die.

**Conclusion** This study demonstrates for the first time that 1,25-(OH)<sub>2</sub>D<sub>3</sub> induces apoptosis in MCF-7 cells by disrupting mitochondrial function, which is accomplished by translocation of Bax to mitochondria, release of cytochrome *c*, production of ROS, and decrease in mitochondrial membrane potential. This is the first data that demonstrates that 1,25-(OH)<sub>2</sub>D<sub>3</sub> signaling on mitochondria does not require caspase activation, since broad-spectrum caspase inhibitor zVAD.fmk was unable to block these mitochondrial events. The failure of 1,25-(OH)<sub>2</sub>D<sub>3</sub> to disrupt mitochondrial function in MCF-7<sup>D3Res</sup> cells suggests that 1,25-(OH)<sub>2</sub>D<sub>3</sub> signaling on mitochondria is a complex event requiring more than a functional VDR. Events upstream of Bax translocation to mitochondria in response to 1,25-(OH)<sub>2</sub>D<sub>3</sub> are abrogated in the vitamin D<sub>3</sub>-resistant cells, and contributed to resistance to 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis. Caspases act solely as executioners by facilitating 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis, but caspase activation is not required for induction of cell death by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in MCF-7 cells. Although caspase inhibitor blocked all the biochemical changes associated with caspase activation occurring following perturbation of mitochondria and loss of cytochrome *c*, the commitment of MCF-7 cells to 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediated apoptosis is caspase independent.

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Supported by NIH (#CA69700) & DAMD (#17-97-1-7183)

Presented at the 83<sup>rd</sup> Annual Meeting of the Endocrine Society in Denver, CO, June, 2001

**DISRUPTION OF MITOCHONDRIAL ACTIVITY AND INDUCTION OF APOPTOSIS BY 1,25-DIHYDROXYVITAMIN D<sub>3</sub> IN MCF-7 BREAST CANCER CELLS.** CJ Narvaez,<sup>1</sup> M Brown,<sup>2</sup> and J Welsh.<sup>1</sup> <sup>1</sup>*Dept. of Biological Sciences, University of Notre Dame, Notre Dame, Indiana;* <sup>2</sup>*Walther Cancer Institute, University of Notre Dame, Notre Dame, Indiana*

Vitamin D<sub>3</sub> compounds offer an alternative approach to anti-hormonal therapies for human breast cancer. Our lab has shown that 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>) induces apoptosis in MCF-7 cells by disruption of mitochondrial function which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species (ROS). These mitochondrial effects of 1,25-D<sub>3</sub> do not require caspase activation, since they are not blocked by the cell permeable caspase inhibitor zVAD.fmk. To further support these observations, fluorescence-activated cell sorting of annexin V-FITC was used to obtain populations enriched in viable and apoptotic cells. Loss of cytosolic Bax and enrichment of membrane bound Bax, as well as down-regulation of Bcl-2, was observed in the apoptotic cell population treated with 1,25-D<sub>3</sub>. Redistribution of cytochrome *c* from mitochondria to cytosol was detected within 48 hrs of 1,25-D<sub>3</sub> treatment in MCF-7 cells, prior to the onset of apoptotic morphology. Long-term exclusion of cytochrome *c* from the electron transport chain can lead to ROS generation due to incomplete reduction of molecular oxygen. Time course studies demonstrated that ROS production was enhanced within 72 hrs of 1,25-D<sub>3</sub> treatment in MCF-7 cells whereas loss of mitochondrial membrane potential was a late event during 1,25-D<sub>3</sub> mediated apoptosis. Cytochrome *c* release into the cytosol triggers caspase activity, which leads to substrate cleavage, and externalization of phosphatidylserine. The earliest detection of phosphatidylserine exposure was 96 hrs after treatment with 1,25-D<sub>3</sub> indicating that mitochondrial disruption precedes activation of downstream caspases. Our results demonstrate that 1,25-D<sub>3</sub> mediates apoptosis of MCF-7 cells through mitochondrial signaling and ROS generation, which is regulated by the Bcl-2 family of apoptotic regulators. Caspases act solely as executioners to facilitate 1,25-D<sub>3</sub> mediated apoptosis, and caspase activation is not required for induction of cell death by 1,25-D<sub>3</sub>.

Abstract promoted to a 10 min presentation at **the Eleventh Workshop on Vitamin D** in Nashville, TN, May 27-June 1, 2000.

**ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS.** CJ Narvaez and J Welsh Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-D<sub>3</sub> mediated apoptosis in MCF-7 breast cancer cells *in vitro*. Treatment of MCF-7 cells with 1,25-D<sub>3</sub> induced DNA fragmentation, external display of phosphatidylserine (PS), and cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP). Mitochondrial events associated with 1,25-D<sub>3</sub> mediated apoptosis included translocation of Bax, disruption of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of cytochrome *c*. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D<sub>3</sub> mediated apoptosis. The effect of 1,25-D<sub>3</sub> on MCF-7 cells was compared to that of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), which induces apoptosis via a caspase-dependent pathway. zVAD.fmk prevented 1,25-D<sub>3</sub> mediated PARP cleavage, PS exposure, and DNA fragmentation. In contrast, zVAD.fmk did not prevent 1,25-D<sub>3</sub> mediated redistribution of Bax from cytosol to mitochondria, loss of mitochondrial membrane potential, generation of ROS, or release of cytochrome *c*. In addition, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-D<sub>3</sub> treatment of a MCF-7 cell clone selected for resistance to 1,25-D<sub>3</sub> mediated apoptosis. Most significantly, zVAD.fmk did not protect MCF-7 cells from 1,25-D<sub>3</sub> induced apoptosis, indicating that the commitment of MCF-7 cells to 1,25-D<sub>3</sub> mediated cell death is caspase independent. The sensitivity of 1,25-D<sub>3</sub> induced apoptosis to caspase inhibition was found to be distinct from that of TNF $\alpha$  induced apoptosis, which was completely prevented by zVAD.fmk. In summary, these data clearly indicate that although caspase inhibition can block some of the late stages of 1,25-D<sub>3</sub> mediated apoptosis in MCF-7 cells; the commitment to cell death is caspase independent. Our data implicate Bax distribution and mitochondrial disruption as critical caspase independent events in 1,25-D<sub>3</sub> mediated apoptosis of breast cancer cells. *Supported by NIH CA69700 & DAMD17-97-1-7183.*

Poster presented at the *Era of Hope Department of Defense Breast Cancer Research Program Meeting* in Atlanta, GA, June 8-11, 2000.

**ROLE OF MITOCHONDRIA AND CASPASES IN VITAMIN D MEDIATED APOPTOSIS IN MCF-7 BREAST CANCER CELLS**

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Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, induces apoptosis in breast cancer cells and tumors, but the underlying mechanisms are poorly characterized. In these studies, we focused on the role of caspase activation and mitochondrial disruption in 1,25-D<sub>3</sub> mediated apoptosis in MCF-7 breast cancer cells *in vitro*. Treatment of MCF-7 cells with 1,25-D<sub>3</sub> induced DNA fragmentation, external display of phosphatidylserine (PS), and cleavage of the caspase substrate, poly (ADP-ribose) polymerase (PARP). Mitochondrial events associated with 1,25-D<sub>3</sub> mediated apoptosis included translocation of Bax, disruption of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and release of cytochrome *c*. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (z-Val-Ala-Asp-fluoromethylketone, zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D<sub>3</sub> mediated apoptosis. The effect of 1,25-D<sub>3</sub> on MCF-7 cells was compared to that of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), which induces apoptosis via a caspase-dependent pathway. zVAD.fmk prevented 1,25-D<sub>3</sub> mediated PARP cleavage, PS exposure, and DNA fragmentation. In contrast, zVAD.fmk did not prevent 1,25-D<sub>3</sub> mediated redistribution of Bax from cytosol to mitochondria, loss of mitochondrial membrane potential, generation of ROS, or release of cytochrome *c*. In addition, we show that Bax translocation and mitochondrial disruption do not occur after 1,25-D<sub>3</sub> treatment of a MCF-7 cell clone selected for resistance to 1,25-D<sub>3</sub> mediated apoptosis. Most significantly, zVAD.fmk did not protect MCF-7 cells from 1,25-D<sub>3</sub> induced apoptosis, indicating that the commitment of MCF-7 cells to 1,25-D<sub>3</sub> mediated cell death is caspase independent. The sensitivity of 1,25-D<sub>3</sub> induced apoptosis to caspase inhibition was found to be distinct from that of TNF $\alpha$  induced apoptosis, which was completely prevented by zVAD.fmk. In summary, these data clearly indicate that although caspase inhibition can block some of the late stages of 1,25-D<sub>3</sub> mediated apoptosis in MCF-7 cells, the commitment to cell death is caspase independent. Our data implicate Bax distribution and mitochondrial disruption as critical caspase independent events in 1,25-D<sub>3</sub> mediated apoptosis of breast cancer cells.

The US Army Medical Research and Materiel Command under DAMD17-97-1-7183 supported this work.



Poster presented at the 1999 Keystone Symposia "Apoptosis and Programmed Cell Death" at Breckenridge, CO, April 6-11, 1999.

**Caspase-Independent Apoptosis By Vitamin D Treatment in MCF-7 Breast Cancer Cells.** Carmen J. Narvaez and JoEllen Welsh, Dept of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-D<sub>3</sub>), the active form of vitamin D<sub>3</sub>, is not only a powerful regulator of calcium homeostasis, but is a steroid hormone with important roles in cell growth and differentiation. We have shown that 1,25-D<sub>3</sub> induces morphological and biochemical markers of apoptosis (chromatin and cytoplasmic condensation, and DNA fragmentation) in MCF-7 breast cancer cells. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells, we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D<sub>3</sub> mediated apoptosis. The effects of 1,25-D<sub>3</sub> treatment on MCF-7 cells were compared to TNF $\alpha$  which induces apoptosis by a caspase-dependent mechanism. zVAD-fmk protected MCF-7 cells from TNF $\alpha$  mediated apoptosis but did not protect MCF-7 cells from the effects of 1,25-D<sub>3</sub> treatment. The caspase inhibitor did not block the effects of 1,25-D<sub>3</sub> on cell number, mitochondrial membrane potential, or release of cytochrome c. zVAD-fmk did prevent 1,25-D<sub>3</sub> mediated DNA fragmentation, PARP cleavage, and external display of phosphatidylserine (PS). These studies suggest that the 1,25-D<sub>3</sub> mediated apoptotic events occurring downstream of mitochondrial disruption are blocked by caspase inhibition, but the commitment of MCF-7 cells to 1,25-D<sub>3</sub> mediated apoptosis is caspase independent. *Supported by NIH (#CA69700) & DAMD (#17-7-1-7183).*



DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
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5 Jun 02

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